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THE ISOLATION OF TYPE 5 ADENOVIRUS USING A FLUOROCARBON; COMBINED MORPHOLOGICAL AND BIOLOGICAL STUDIES

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PURIFIED suspensions of both vaccinia virus (Epstein, 1958*a*) and the Rous virus (Epstein, 1958*b*) have recently been prepared from infected chorioallantoic membranes by means of a fluorocarbon treatment based on that of Gessler, Bender and Parkinson (1956). These suspensions were shown to be almost entirely free of formed host-cell components since, when high speed centrifugation was applied to them and samples of the resulting pellets were examined in the electron microscope, only two zones were found, one composed entirely of uniform particles and the other of amorphous gelatinous material (Epstein, 1958*a* and *b*); no other structures were detected. By making biological tests at each step in the centrifugation and electron microscopy procedures, biological activity was shown to be associated with the uniform particles, thus indicating their viral nature (Epstein, 1958*a* and *b*).

Samples of the virus-containing zones of the pellets fixed with permanganate (Luft, 1956) provided ideal material for electron microscopy and permitted the fine structure of vaccinia virus (Epstein, 1958*a*) and the Rous virus (Epstein, 1958*b*) to be investigated. Additional work with the latter agent determined the site and nature of its nucleic acid (Epstein, 1958*c*; Epstein and Holt, 1958) and demonstrated the further suitability of material of this type for study by integrated electron microscopical and cytochemical procedures.

Thus, the various techniques which were used in close combination provided valuable methods for the isolation and identification of viruses and for the study of their morphology and composition. In view of the encouraging results obtained with these methods in the case of vaccinia virus (Epstein, 1958*a* and *d*; Holt and Epstein, 1958) and the Rous virus (Epstein, 1958*b* and *c*; Epstein and Holt, 1958) it was thought that they might usefully be applied to some other agent. An infective virus of classical behaviour and medium size was decided upon so that its structure and composition could be compared with the medium sized tumour-producing Rous virus already investigated.

An adenovirus was accordingly selected and was grown, for the experiments, in HeLa cell cultures in the manner described by Pereira and Kelly (1957).

Although it was known that adenovirus from tissue cultures maintained its complement-fixing titre unimpaired after two treatments with fluorocarbon but not after more, and that one such treatment removed serologically active host antigens (Hamparian, Müller and Hummeler, 1958), no information was available regarding the state of the viral antigen after the treatments, or the presence or absence of serologically inactive associated host substances. As a first step, therefore, an investigation was made into the purifying effect on adenovirus of treatment with fluorocarbon and this was linked with biological experiments designed to identify the particles isolated. The methods used were based on those developed for earlier work (Epstein, 1958*a*) and the present communication reports the results which have been obtained ; the findings of integrated electron microscopical and cytochemical studies on the structure and composition of the adenovirus are presented elsewhere (Epstein, 1959 ; Epstein, Holt and Powell, 1960).

MATERIALS AND METHODS

Virus strain.—Type 5 adenovirus of the prototype strain has been used ; it was received, through the kindness of Dr. H. G. Pereira, in pooled HeLa cells from infected cultures showing advanced cytopathic effects and has subsequently been passed in stock cultures of these cells.

Stock HeLa cell cultures.—Stock cultures of HeLa cells were grown in flat 8 oz. medical bottles (United Bottle Manufacturers Ltd., London, W.C.2) with rubber stoppers, and when confluent sheets of cells had developed, were used either as a source of fresh cultures or for the propagation of virus. The methods and media employed were essentially those described by Pereira and Kelly (1957), their Medium I being used for growing the cells and Medium III for maintaining them after infection with the virus ; the bottles, however, were incubated at 37° in a horizontal position and the balanced saline solution used throughout differed in that 0·1 g. phenol red, 0·25 g. benzylpenicillin, 0·25 g. streptomycin and 0·125 g. sodium bicarbonate were incorporated in each litre.

Suspending fluid.—The composition of the suspending fluid used for preparing the stock virus and in the titrations has been described elsewhere (Epstein, 1958*a*).

Stock virus.—Batches of stock virus were prepared from time to time during the course of the present series of experiments. For each batch, pooled infected HeLa cells from 6 to 8 stock cultures with marked cytopathic changes were treated with fluorocarbon in the manner

EXPLANATION OF PLATES

All the figures are electron micrographs of sections cut through various regions of pellets prepared from fluorocarbon-treated suspensions of adenovirus.

FIG. 1.—Survey picture of a typical area in the virus-containing portion of the dense white zone of a pellet. Uniform virus particles 60 mμ in diameter are the only formed structures present. ×20,000.

FIG. 2.—Part of the virus-containing zone in which some of the particles are apparently arranged in crystalloid array. ×25,000.

FIG. 3.—Small area of the typical virus-containing part of a pellet showing its composition in greater detail. The virus can be seen to have a hexagonal profile and to contain a central electron dense nucleoid surrounded by a less dense viroplasm. ×60,000.

FIG. 4.—Round elements from the thin basal stratum of the dense white zone ; virus particles are scattered amongst the debris. ×12,500.

FIG. 5.—Membranous structures, perhaps microsomes, from a different part of the same layer as that shown in Fig. 4. Scattered virus particles are also apparent here. ×12,500.

FIG. 6.—Survey picture of a typical part of the gelatinous region of a pellet. ×10,000.

FIG. 7.—Detail of a small area of the gelatinous zone of a pellet. Beaded threads can be readily seen. ×80,000.

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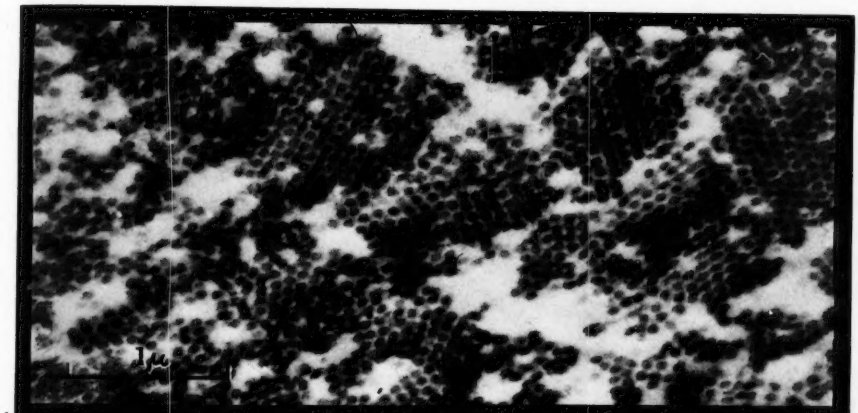
Epstein and Powell.



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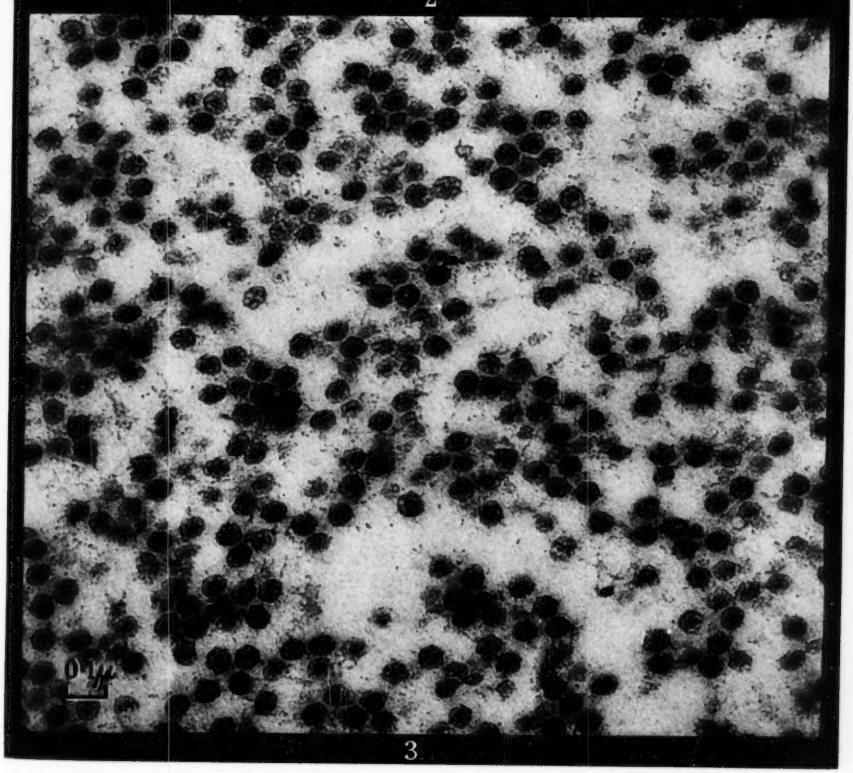

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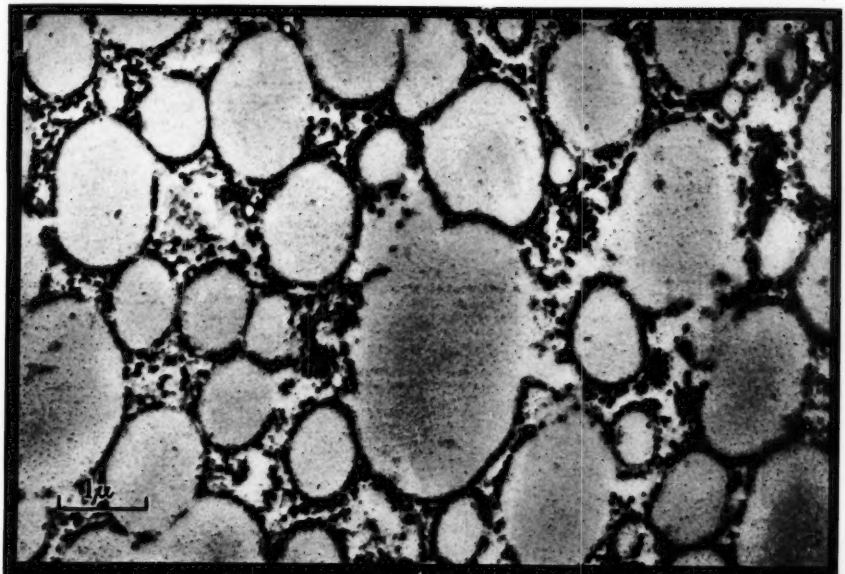

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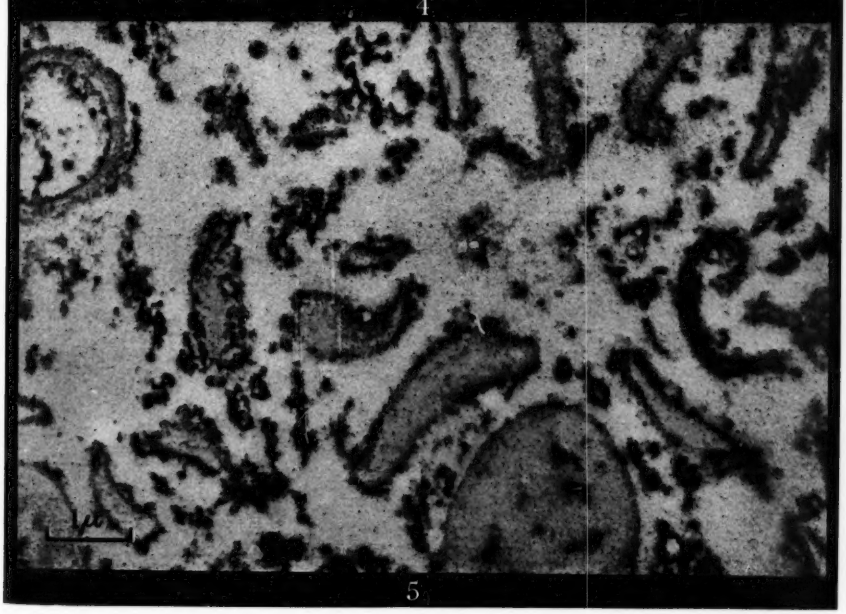

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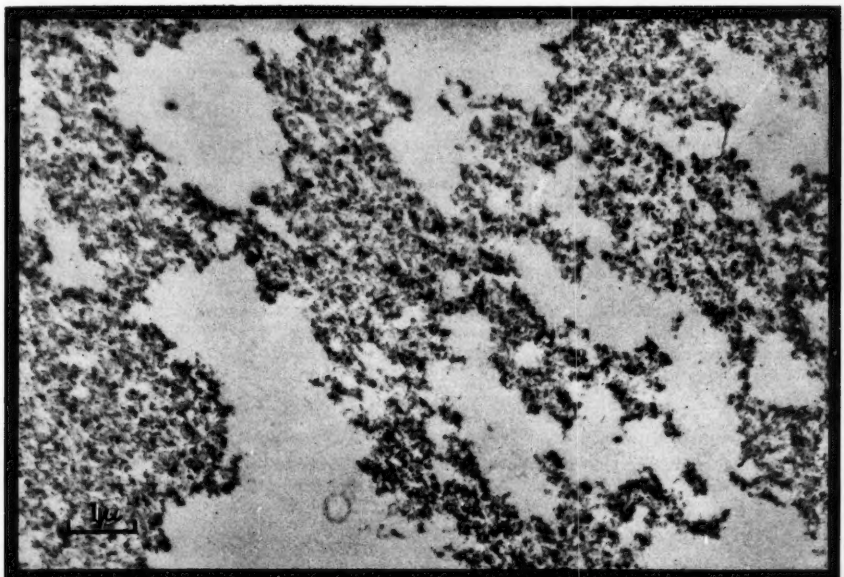

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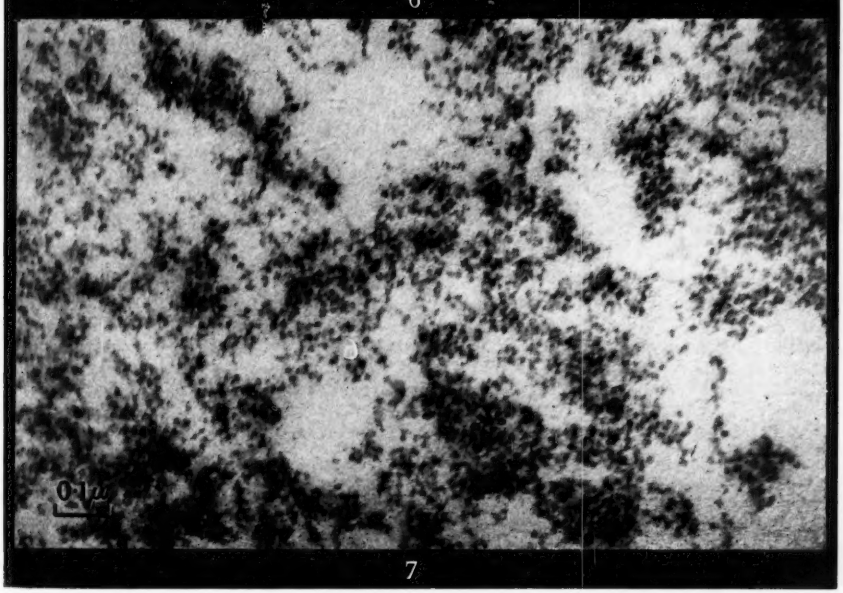

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Epstein and Powell.

described below. The virus-containing watery layer from either the first or the third treatment was mixed with suspending fluid, it being found convenient usually to use equal volumes. The resulting mixture was taken as the stock virus and stored in sealed hard glass ampoules in volumes of about 1 ml. at −70° until required.

Preparation of fluorocarbon-treated suspensions.

For each experiment the thawed contents of an ampoule of stock virus were added to 100 ml. of Medium III (Pereira and Kelly, 1957) which was then divided between 6 to 8 stock HeLa cultures containing confluent sheets of cells. The infected cultures were incubated in a horizontal position for 3 days at 37°, after which advanced macroscopic cytopathic changes were present and detachment of some parts of the cell sheets was usually beginning. Slight shaking of the bottles detached the remainder of the cells which were then harvested by centrifuging the pooled whole cultures at 300 *g* for 2 min. and decanting the supernatant culture fluid from above the pellets of lightly packed cells.

These pellets of cells were treated with 3 applications of a fluorocarbon as previously described (Epstein, 1958*d*). The fluorocarbon used was Arcton 113 (CF_2Cl-CCl_2F) of Imperial Chemical Industries, Ltd., London, who, until recently, designated this same compound as Arcton 63; the aqueous layer from the third application of Arcton constituted the fluorocarbon-treated suspension.

Preparation of pellets for electron microscopy from fluorocarbon-treated suspensions.

The suspensions were subjected to high speed centrifugation and samples were taken from the resulting pellets for permanganate fixation (Luft, 1956), dehydration, embedding and sectioning for electron microscopy, as previously described (Epstein, 1958*a*).

Methods used in assay of virus

Taking of samples.—For bio-assay, samples of fluids and pellets were taken and handled before inoculation as previously described (Epstein, 1958*a*).

Inoculations into HeLa cell tube cultures.—Inoculations were made into tube cultures of HeLa cells prepared 24 hr. in advance by the techniques of Pereira and Kelly (1957) in their fluid Medium II. The inoculum consisted of 0·1 ml. and 4 tubes were used for each dilution of a sample. After inoculation, rabbit serum in balanced saline was added to each tube (Pereira and Kelly, 1957) so that the final contents were equivalent to Medium III, and the tubes were then incubated at 37° and inclined in racks at 5° to the horizontal for 28 days. During this time the medium was changed on the 7th, 14th and 21st days and the tubes were examined every 3 or 4 days for cytopathic changes whose presence or absence was recorded.

General considerations

The various precautions and technical points discussed previously (Epstein, 1958*a*) were taken into account for the present work.

The biological parts of the experiments were done with strict aseptic technique; tests for the presence of contaminating bacteria were negative.

For the electron microscopy, a Philips electron microscope (EM 100) was used with a high resolution objective lens, 30μ objective aperture and flat Wehnelt cylinder. Sections were mounted on carbon coated copper grids (Watson, 1956) and were examined without removal of the embedding medium; electron micrographs were taken using an accelerating voltage of 60 kv.

Experimental procedure

Experiments 1 and 2.—In each of these experiments a pellet was made from a fluorocarbon-treated suspension; samples were then taken from all areas of the pellet and were prepared for electron microscopy. The samples were investigated with the electron microscope by examining serial sections cut from them at various levels.

Experiments 3 to 7.—In these further experiments, pellets were prepared and treated as before for morphological investigation. Meanwhile samples were taken for bio-assay from the fluorocarbon-treated suspension, the supernatant fluid above the pellet (Spinco supernatant) and from the fluids in which samples cut from various regions of the pellets had been re-suspended (Epstein, 1958*a*). All these samples were diluted in serial 10-fold steps with

suspending fluid using a clean pipette for each dilution, and portions of the various dilutions were inoculated into tube cultures of HeLa cells.

Calculation of results

From the number of tubes of HeLa cells which developed cytopathic changes following the inoculation of each dilution of a sample (Table), that dilution of the sample which would have caused the changes in 50 per cent of the tubes when given in a dose of 1 ml. was calculated. The method of calculation used was that of Reed and Muench (1938) and the results have been recorded in the Table under the heading of Lesion Dilution 50 (LD50).

RESULTS

Macroscopic appearance of the pellets

The pellets measured about 4–5 mm. diameter and had an appearance which was somewhat similar to that of pellets prepared in the same manner from chorioallantoic membranes infected with vaccinia or Rous virus (Epstein, 1958*a*, *b*). With the present material, however, the ring was faint and less sharply demarcated, the tongue likewise faint, and the dense white zone abundant; it frequently exceeded 2 mm. in diameter and extended into the base of the tongue. The rest of the pellet consisted of transparent, moderately firm jelly covering and merging into the other regions, just as was the case with pellets prepared from vaccinia and Rous virus-infected material.

Electron microscopy of the pellets

Dense white zone.—The dense white zone consisted almost entirely of uniform particles measuring about 60 mμ in diameter; the particles were mostly scattered evenly at random (Fig. 1), but aggregation in arrays which were possibly crystalline was also observed in the lower layers of the zone (Fig. 2). At higher magnification the particles were seen to be hexagonal in outline and to contain a central electron dense nucleoid surrounded by a less dense outer zone or viroplasm (Fig. 3). In the extensive area occupied by the particles no other formed elements were observed (Fig. 1, 2 and 3), but below this, forming a very thin basal layer to the dense white zone, cell debris was found. It consisted in some parts of round, relatively large, elements (Fig. 4), and in other parts of membrane bounded structures (Fig. 5); 60 mμ particles were scattered in both types of debris (Fig. 4 and 5).

Gelatinous zones.—The jelly, which composed the rest of the pellet, consisted of uniform fuzzy material arranged in strands (Fig. 6). In the lower layers of jelly from the ring and tongue areas these strands showed some packing. At high magnification the jelly was observed to be made up of fine beaded threads (Fig. 7).

Combined electron microscopy and titrations

The Table shows the results of the experiments in which the biological activity of samples taken at various stages during the preparation of pellets was investigated and related to the morphological observations made with the electron microscope on samples from different regions of each pellet.

It is evident that the infectivity of each suspension was very much greater than that of the supernatant fluid (Spinco supernatant) above the pellet resulting from high speed centrifugation of the suspension (Table). After such centrifuga-

TABLE—*The Biological Activity of Samples Taken at Different Stages During the Preparation of Pellets from Fluorocarbon-treated Suspensions (Titration in Tube Cultures of HeLa Cells)*

Expt. No.	Sample	Dilutions of sample and number of tubes with cytopathic changes from 4 inoculations of each dilution										LD_{50} per 1-ml. sample
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	
3	Fluorocarbon-treated suspension	—	—	4/4	4/4	4/4	4/4	3/4	2/4	0/4	0/3*	$10^{-8.8}$
	Spinco supernatant	—	—	4/4	4/4	3/4	0/4	0/4	—	—	—	$10^{-6.3}$
	White pellet	—	—	4/4	1/4	0/4	0/4	0/4	0/4	—	—	$10^{-4.6}$
4	Fluorocarbon-treated suspension	—	—	—	4/4	4/4	2/4	0/4	0/4	0/4	0/4	10^{-7}
	White jelly	—	4/4	4/4	0/4	0/4	0/4	0/4	—	—	—	$10^{-4.5}$
	Pellet jelly	4/4	4/4	0/4	0/4	0/4	—	—	—	—	—	$10^{-3.5}$
5	Fluorocarbon-treated suspension	—	—	—	—	4/4	4/4	4/4	1/4	1/4	0/4	$10^{-8.8}$
	Spinco supernatant	—	—	4/4	4/4	4/4	0/4	0/4	—	—	—	$10^{-6.5}$
	White pellet	—	4/4	2/4	1/4	0/4	0/4	0/4	—	—	—	$10^{-4.2}$
	Pellet jelly	4/4	2/4	0/3*	0/4	0/4	—	—	—	—	—	10^{-3}
6	Fluorocarbon-treated suspension	—	—	—	4/4	4/4	4/4	3/4	1/4	0/4	0/4	$10^{-8.5}$
	Spinco supernatant	—	—	4/4	2/4	2/4	0/4	0/4	0/4	—	—	$10^{-5.5}$
	White pellet	—	3/4	2/4	0/4	0/4	0/4	0/3*	—	—	—	$10^{-3.8}$
	Pellet jelly	4/4	1/4	1/4	0/4	0/4	—	—	—	—	—	$10^{-2.8}$
7	Fluorocarbon-treated suspension	—	—	—	4/4	4/4	4/4	1/4	0/4	0/4	0/4	$10^{-7.7}$
	Spinco supernatant	—	—	3/4	1/4	1/4	0/4	0/4	0/4	—	—	$10^{-4.7}$
	White pellet	—	4/4	4/4	4/4	3/4	1/4	0/4	—	—	—	$10^{-6.5}$
	Pellet jelly	4/4	4/4	4/4	4/4	1/4	—	—	—	—	—	$10^{-5.7}$

* Culture tubes which developed bacterial contamination during incubation were discarded.

tion 99·9 per cent of the biological activity was eliminated from the suspensions on two occasions (Expt. 6 and 7) and slightly less than this on two others (Expt. 3 and 5). That this activity was deposited in the white zones of the pellets is shown by the fact that a very small sample from this zone was highly active (Expt. 3–7) even though it had been re-suspended in as great a volume of fluid (Epstein, 1958*a*) as the original suspension from which the whole pellet came; in contrast, similarly re-suspended samples from the gelatinous zones of the pellets possessed considerably less activity (Expt. 4–7).

When samples taken from the white zones of the pellets were examined in the electron microscope they were seen to consist entirely of uniform hexagonal particles about 60 mμ in diameter (Fig. 1, 2 and 3) except for the cell debris forming the very narrow basal stratum below (Fig. 4 and 5). Samples of the gelatinous areas of the pellets were found to be free of such particles (Fig. 6 and 7) when examined in this way.

DISCUSSION

Although three applications of fluorocarbon (Epstein, 1958*a*) were used in preparing suspensions for the present experiments, free virus in such suspensions was only exposed to the fluorocarbon twice in the free state, since the second treatment was in fact a re-extraction of the original cellular starting material. The procedure followed thus took into account the findings of Hamparian,

Müller and Hummeler (1958) regarding the impairment of the specific complement-fixing antigen of adenovirus by more than two treatments with fluorocarbon.

The nature of the large round elements found (Fig. 4) is not known, but the membrane bounded structures (Fig. 5) could, despite their relatively large size, be microsomes. The latter are now considered to be derived by fragmentation from the rough surfaced elements of the endoplasmic reticulum (Palade, 1958) and the unusual largeness of the structures observed here might simply be a result of the type of fragmentation produced by the present preparation procedure. The finding of apparently smooth limiting membranes around the structures (Fig. 5) is not inconsistent with their identification as microsomes, since it has been known for some time that the characteristic 15 mμ ribonucleoprotein particles which are seen, after osmium fixation, studding the outside of microsomal membranes and causing their "rough" appearane (Palade and Siekevitz, 1956*a* and *b*), are not preserved in material fixed with permanganate (Luft, 1956).

That a layer of cell debris was found in the pellets obtained from fluorocarbon-treated suspensions of HeLa cells infected with adenovirus, is in marked contrast to the absence of such debris in the material obtained previously from similarly treated vaccinia-infected chorioallantoic membranes (Epstein, 1958*a*). This difference is in all probability due to variation in the number of applications of fluorocarbon used in the two sets of experiments—namely three in the present series as against five in the work with vaccinia—and support for such an explanation is afforded by the results of earlier studies on fluorocarbon purification of the Rous virus. In these it was found that pellets derived from egg grown Rous nodules treated five times with the fluorocarbon contained membranous cell debris, whereas those prepared after seven treatments did not (Epstein, 1958*b*). Elimination of formed host cell components would thus appear to depend on the number of times fluorocarbon is applied and this number seems, in turn, to require variation both with the relative quantities of cells and reagents involved and with each particular cell system. The present findings taken together with the results of the work with the Rous virus (Epstein, 1958*b*) further suggest that membranous microsomal elements are the last cell structures to be removed in the course of repeated applications of fluorocarbon.

The presence of jelly in the pellets shows that fluorocarbon treatment failed to remove certain unformed contaminating host substances, just as was found to be the case earlier in the vaccinia and Rous virus experiments (Epstein, 1958*a* and *b*), and when examined in the electron microscope, the jelly from each system was morphologically similar.

From the results of the combined morphological and biological experiments it is evident that the fluorocarbon treatment used enabled suspensions to be made in which the only formed structures were vast numbers of uniform polyhedral particles 60 mμ in diameter and hexagonal in profile, together with a very small quantity of cellular debris. This follows from the fact that when high speed centrifugation was applied to the suspensions, the resulting pellets consisted only of particles (Fig. 1, 2 and 3), amorphous fuzzy material (Fig. 6) and the thin layer of cell fragments at the base of the dense white zone (Fig. 4 and 5).

The identity of the particles is indicated by the results of the titration experiments (Table). These show that when the suspensions were centrifuged their infectivity came to lie in that region of each pellet occupied only by particles and

a thin layer of cell fragments. Now, similar cell fragments without infectivity have been isolated from uninfected HeLa cells by the same techniques as those used here, whilst particles have not (Holt, Epstein and Powell, unpublished) and work reported by Pereira and Valentine (1958) has clearly shown that infection with adenovirus depends on the presence of particles, even as few as 10 being capable of forming an infective dose. The particles in question were of the same order of size as those observed here in the infective white zones of pellets, and indeed the correspondence is very close if allowance is made for flattening during the air-drying of the preparation method which Pereira and Valentine (1958) used. Thus, the uniform 60 mμ particles which formed the bulk of the white zones appeared similar to the adenovirus in many respects and were associated with a similar type of infectivity.

A further reason for concluding that the particles were in fact the adenovirus is provided by the fact that such particles have been observed with the electron microscope in adeno-infected tissue culture cells by many workers (Kjellén, Lagermalm, Svedmyr and Thorsson, 1955; Morgan, Howe, Rose and Moore, 1956; Harford, Hamlin, Parker and van Ravenswaay, 1956), both scattered in the nuclei and also frequently arranged there in striking, apparently crystalline, arrays, very similar to those found in the pellets (Fig. 3). Although these virus crystalloids in the pellets might have arisen after extraction and purification of the particles, it is more likely that they represent intact portions of crystals present in the nuclei of the original infected HeLa cells used as starting material for each of the present experiments.

The conclusion that infectivity was associated with the particles is also supported by the similar results obtained in earlier experiments made with vaccinia and the Rous virus using the same technical approach (Epstein, 1958*a* and *b*); because of the uniform findings the validity of any one set of experiments is enhanced by the others.

In this connection it should be noticed that the difference in infectivity between samples of the gelatinous and white zones of adeno pellets was much the same as that found in Rous pellets (Epstein, 1958*b*), and much less than that in vaccinia pellets (Epstein, 1958*a*). The difficulty of avoiding clumping and aggregation when samples of the relatively small Rous virus were re-suspended for titrating has already been pointed out (Epstein, 1958*b*) and it would seem that the same difficulty has now been encountered with the similarly sized adenovirus.

Besides indicating the viral nature of the particles from adenovirus-infected tissue cultures, the present work shows that, by the techniques used, these particles can be obtained entirely free from formed host elements in a circumscribed area of pellet (Fig. 1). When isolated in this way the particles have still been morphologically intact (Fig. 1, 2 and 3) and associated with infectivity (Table) and it seems probable that they could be freed from the other components in the pellets by density gradient centrifugation, or adsorption on and elution from, appropriate separating columns.

However, even without any further treatment, the purified particles in the white zones of pellets provide admirable material for the study of virus structure and composition. This has already been demonstrated in the case of vaccinia (Epstein, 1958*a*) and the Rous virus (Epstein, 1958*b* and *c*; Epstein and Holt, 1958) and recent investigations reported elsewhere (Epstein, Holt and Powell, 1960) now add confirmation with adenovirus.

SUMMARY

An investigation into the purifying effect of a fluorocarbon (CF_2Cl-CCl_2F) on adenovirus grown in HeLa cell cultures is described. Pellets made by high speed centrifugation of treated virus suspensions have been prepared for sectioning for electron microscopy so that their purity could be assessed by direct observation; this morphological work was combined with tests for biological activity.

The results show that the fluorocarbon treatment yielded suspensions which contained amorphous host substances, a small amount of formed cellular debris and very large quantities of uniform 60 mμ particles; the latter were shown in the biological tests to be associated with infectivity, thus indicating their viral nature.

Both the reasons for concluding that the cell debris is unassociated with infectivity and the significance of this material in relation to fluorocarbon purification are discussed.

The usefulness of the extensive zone in the pellets in which purified virus was found isolated from all formed host debris is also considered.

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THE FINE STRUCTURE AND COMPOSITION OF TYPE 5 ADENOVIRUS; AN INTEGRATED ELECTRON MICROSCOPICAL AND CYTOCHEMICAL STUDY

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CURRENT interest in the morphology and composition of viruses has been much influenced recently by the fact that data regarding structure and composition lead directly to the consideration of such fundamental questions as the basic biological nature of viral agents, the manner in which they replicate, and the mechanisms whereby their effects are produced.

Viruses of the adeno group have received considerable attention in this connection following the demonstration by Kjellén, Lagermalm, Svedmyr and Thorsson (1955) of intranuclear virus-like particles in crystalloid array in sections of infected tissue culture cells examined with the electron microscope. This provided the first example of an animal virus so arranged *in vivo*, and the finding has subsequently been confirmed and amplified (Harford, Hamlin, Parker and van Ravenswaay, 1956; Morgan, Howe, Rose and Moore, 1956; Lagermalm, Kjellén, Thorsson and Svedmyr, 1957). The individual particles have been described as being round or ellipsoid when seen in sections and they are known to contain a dense inner body or nucleoid surrounded by a less dense outer zone or viroplasm (Harford, Hamlin, Parker and van Ravenswaay, 1956; Lagermalm *et al.*, 1957; Morgan and Rose, 1959). However, particles dried from suspension and examined as whole mounts have revealed a hexagonal outline (Tousimis and Hilleman, 1957; Valentine and Hopper, 1957) and this discrepancy has been ascribed by some workers to an artefact caused by drying (Morgan and Rose, 1959).

When the Feulgen reaction was applied to infected cell nuclei it was found that those regions shown by electron microscopy to be occupied by virus-like particles gave an intensely positive response for deoxyribonucleic acid (DNA) (Bloch, Morgan, Godman, Howe and Rose, 1957), and because of this it has been assumed that adenoviruses are of DNA type (Bloch *et al.*, 1957; Morgan and Rose, 1959). However, the positive Feulgen reaction could well have been due to host cell material lying between the particles rather than to a component of the particles themselves and it is in fact known that a great increase in cellular DNA does occur during adenovirus infection (Ginsberg and Dixon, 1959). There is also evidence that DNA can be present in large amounts without being detectable in sections with the electron microscope (Holt and Epstein, 1958), the Feulgen stain not being visible, of course, by this means. But whatever the nature of the nucleic acid in the adenovirus, it seems probable, as was suggested recently (Pereira, 1959), that it might be concentrated in the dense central nucleoid, presumably in the same manner as was found to be the case with the Rous virus (Epstein, 1958*a*).

Now in the course of earlier studies with vaccinia and the Rous virus, it was shown that when these agents were fixed with potassium permanganate for electron microscopy (Luft, 1956), excellent preparations were regularly obtained and important new structures were revealed (Epstein, 1958*b*, *c* and *d*). Furthermore, such fixation did not interfere with the subsequent digestion of nucleic acids by specific nucleases (Epstein, 1958*a*; Holt and Epstein, 1958) and integrated electron microscopical and cytochemical investigations based on this yielded valuable information concerning the morphology and composition of the Rous virus (Epstein and Holt, 1958).

It was considered that similar methods might usefully be applied to the adenovirus particle and as a first step the isolation and identification of this agent in suitable pellets was undertaken. A fluorocarbon separation procedure was used and pellets were obtained in which a dense white zone consisting of virus particles was found (Epstein and Powell, 1960).

In the first part of the present investigation samples from the virus-containing zones of pellets (Epstein and Powell, 1960) were fixed with permanganate (Luft, 1956) and prepared for electron microscopy in order to study the fine structure of the adenovirus after such treatment; a brief preliminary account of this has been given (Epstein, 1959). In further work, the effects of specific digestions on the morphology of the adenovirus have been assessed with the electron microscope and integrated with the results of parallel cytochemical studies. Both parts of this investigation are described in detail here.

MATERIALS AND METHODS

Preparation of pellets.—Suspensions of Type 5 adenovirus were made from infected HeLa cell cultures by 3 treatments with a fluorocarbon, and pellets were then prepared from them; the methods used have been described elsewhere (Epstein, 1958*c*; Epstein and Powell, 1960).

Electron microscopy.—Samples were taken from the virus-containing white zones of the pellets and were fixed, embedded, sectioned and examined with the electron microscope as in previous work (Epstein, 1958*c*; Epstein and Powell, 1960). In addition, samples from the pellets were fixed for about 1 hr. as before, or for 15 min., taken through 30 per cent ethanol until they reached room temperature (Luft, 1956) and were then subjected to various digestive treatments before being prepared for electron microscopy. The methods of treatment and the controls used are described below.

Cytochemical tests

Taking of samples.—Samples from the virus-containing portions of the pellets, taken exactly as those used for electron microscopy, were smeared in streaks on to spirit-cleaned microscope slides with a lachrymal sac knife and were allowed to dry at room temperature.

Staining for nucleic acids.—Acridine orange staining for the fluorescence microscopy of nucleic acids was done on the dried smears of pellets fixed either in Carnoy's fluid or in 4 per cent acetic acid saturated with mercuric chloride.

In addition, this staining was done on dried smears which had been fixed and then subjected to digestive treatments. Where the latter included the use of deoxyribonuclease (DNase), Carnoy's fixative was applied, and where ribonuclease (RNase), mercury acetic. The digestive treatments and their controls are set out below, whilst the methods used for staining, examining and photographing the preparations by ultra-violet light (UVL) were those described earlier (Epstein and Holt, 1958).

Digestive treatments

All the treatments were applied for 2 hr. at 37° except in the case of Expt 5 (Table I) where one incubation in the sequence was continued for only 70 min. The incubation media were aqueous and contained ethanol to a final concentration of 30 per cent. Controls for the enzyme digestions were incubated in the appropriate enzyme-free medium.

RNase.—The enzyme was used at a concentration of 1 mg. per ml. and at pH 7·0.

DNase.—This was used under the same conditions as RNase except that the solution also contained magnesium chloride at a concentration of 0·003 M.

DNase II (Acid DNase).—In this case the enzyme was used at a concentration of 0·5 mg. per ml. in 0·1 M acetate buffer at pH 5·0, containing a suitable concentration of magnesium chloride (0·001 M) (Oth, Fredericq and Hacha, 1958); the enzyme was generously supplied to us by Professor E. Fredericq.

Pepsin.—This enzyme was applied at a concentration of 0·2 mg. per ml. in 0·01 N hydrochloric acid (pH 2·0).

Alkaline buffer.—Michaelis veronal buffer pH 9·0 was used.

Experimental procedure

In each experiment a fluorocarbon-treated suspension of adenovirus was subjected to high speed centrifugation and a pellet obtained from it. Samples were taken from the virus-containing white zone of the pellet and were prepared for electron microscopy or cytochemical testing; in both cases preparation was done either directly, or after the application of one or more digestive treatments, or after appropriate control treatments. The sequences of treatments and controls used in the various experiments are shown in the Tables.

RESULTS

Electron microscopy

Morphology of the virus particle examined directly.—When prepared directly and examined in the electron microscope the adenovirus was found to be about 60 mμ in diameter and hexagonal in profile (Fig. 1). Varying amounts of individual particles were of course present in a given section, and those cut centrally showed a central electron-dense nucleoid surrounded by a less dense viroplasm (Fig. 1); in addition, a fine outer limiting membrane seemed to be present. The nucleoids had a diameter of about 35 mμ whilst the surrounding viroplasm of low density was about 12 mμ wide, the whole particle thus being about 60 mμ across (Fig. 1).

The examination of very thin sections at high magnification showed that the nucleoid contained a threadlike component apparently rolled up into a ball in a haphazard manner (Fig. 2), that the limiting membrane around the particle was very insubstantial, and that the surface was smooth (Fig. 2 and 3); in such material the hexagonal outline of the virus particle was particularly striking. In favourably orientated sections of extreme thinness the threadlike element in the nucleoid could be seen arranged in parallel curving array and a second nucleoidal component consisting of diffuse dense material could be distinguished (Fig. 3).

Effects of digestive treatments on virus morphology.—All the digestive treatments, irrespective of any specific effects, made the particles look somewhat ragged and extracted by comparison with those prepared directly, and the greater the number of treatments the more this was noticeable. Nevertheless, even after 4–6 hr. incubation in watery medium the essential features of the virus remained intact, the density of the nucleoid being preserved and even appearing emphasized in contrast to the extracted viroplasm surrounding it (Fig. 4, 6 and 7). Another result of this non-specific extraction was to disclose a beaded appearance of the viroplasm accompanied by irregularity in the usually smooth surface of the virus (Fig. 4).

The effects on the morphology of the nucleoid of the digestive treatments applied in the various experiments are summarized in Table I. It can be seen that the nucleoid remained unaffected by RNase, DNase, pepsin and the acidic medium in which the pepsin was dissolved (Expt 1 and 2). Both RNase and DNase were likewise ineffective when applied after preliminary treatment with pepsin (Expt 3),

TABLE I.—*The Effects of Various Digestive Treatments on the Morphology of the Nucleoid of Permanganate-fixed Adenovirus as Observed with the Electron Microscope. Treatments applied for 2 hr. Except where Indicated*

Expt. No.	Fixation time (min.)	Treatments applied to pelleted virus			Effect on nucleoid
		1st	2nd	3rd	
1*a*	60	RNase	—	—	None
b	60	RNase control	—	—	,,
2*a*	60	Pepsin	—	—	,,
b	60	Pepsin control	—	—	,,
c	60	DNase	—	—	,,
d	60	DNase control	—	—	,,
3*a*	60	Pepsin	RNase	—	,,
b	60	,,	RNase control	—	,,
c	60	,,	DNase	—	,,
d	60	,,	DNase control	—	,,
4*a*	50	,,	DNase	Pepsin	,,
b	50	,,	,,	Pepsin control	,,
c	50	,,	RNase	Pepsin	,,
d	50	,,	,,	Pepsin control	,,
5*a*	55	,,	DNase II*	Pepsin	,,
b	55	,,	DNase II control*	,,	,,
c	55	,,	DNase II*	Pepsin control	,,
6*a*	15	,,	DNase	Pepsin	,,
b	15	,,	DNase control	,,	,,
7*a*	15	Buffer at pH 9	—	—	,,
8*a*	15	,, ,, ,,	RNase	—	,,
b	15	,, ,, ,,	DNase	—	Digested
c	15	,, ,, ,,	DNase control	—	None

* Treatment applied for 70 min.

or when employed in this way and followed by a further pepsin treatment (Expt 4). DNase II used in a similar sequence also failed to cause recognizable morphological changes (Expt 5). In all these experiments the virus had been fixed for about 1 hr.; the nucleoid of virus fixed for only 15 min. was equally resistant to the consecutive applications of pepsin, DNase and further pepsin (Expt 6).

In marked contrast to this, virus particles which had been subjected to DNase digestion following preliminary incubation in buffer at pH 9·0 (Expt 8b) had their nucleoids removed and showed an empty space in the central area (Fig. 5). Control particles incubated in the alkaline medium and then subjected to RNase or enzyme-free DNase medium (Expt 8a and c), showed no change in the density of their nucleoids (Fig. 6 and 7). In addition the particles exposed to the alkaline buffer appeared slightly swollen (Fig. 6 and 7), those subsequently digested with DNase showing the greatest degree of enlargement (Fig. 5).

Cytochemical tests

After direct acridine orange staining the virus smears exhibited an orange-red fluorescence irrespective of whether they were fixed in Carnoy's fluid or in the mercury-containing fixative (Fig. 8). A similar orange-red fluorescence was

obtained with appropriately fixed smears that had been digested with RNase (Fig. 9) or DNase before staining with acridine orange. When however the smears were previously incubated with veronal buffer for 2 hr at pH 9·0, and then stained with acridine orange, the orange fluorescence was abolished and replaced by the green fluorescence illustrated in Fig. 10. When smears that had been incubated in the alkaline buffer for 2 hr. were subsequently incubated with DNase for a further 2 hr. and then stained with acridine orange, the fluorescence response was almost completely abolished ; certainly no trace of green fluorescence was seen, but only a barely perceptible orange colour (Fig. 11). Control preparations incubated in the alkaline buffer and then treated with enzyme-free medium alone before the staining showed the same green fluorescence as those in which staining was done directly after incubation at pH 9·0 (Fig. 10). These results are summarized in Table II which also shows the results of the parallel electron microscopy

TABLE II.—*The Effects of Various Treatments on the Fluorescence Response in UVL Given by Smears of Pelleted Adenovirus Stained with Acridine Orange. The State of the Viral Nucleoid Observed by Electron Microscopy After the Treatments, is Shown in Parallel. All Treatments Applied for 2 hr.*

Expt. No.	Treatments applied to smears of virus before staining		Fluorescence response (UVL)	State of nucleoid after treatment (electron-microscopy)
	1st	2nd		
9*a*	—	—	Red	Intact
b	RNase	—	,,	Intact (1*a*)*
c	DNase	—	,,	Intact (2*c*)*
d	Buffer at pH 9	—	Green	Intact (7*a*)*
e	,, ,, ,,	DNase	Abolished	Digested (8*b*)*
f	,, ,, ,,	DNase control	Green	Intact (8*c*)*

* Numbers in brackets refer to the experiments listed in Table I.

experiments, taken from those given in Table I, in order to bring out the correspondence between the two sets of findings. It should be noted that the true colours of the fluorescence responses are only approximately reproduced in Figs. 8–11.

DISCUSSION

Most of the considerations underlying the technical aspects of the present work have already been discussed (Epstein, 1958*c* ; Epstein and Holt, 1958 ; Epstein and Powell, 1960), but an important point arising from the use of permanganate fixation for electron microscopy calls for further comment. It concerns the fact that all the digestive treatments were applied in a medium of 30 per cent ethanol. Luft (1956), in his original description of the potassium permanganate fixative, recommended a specific series of alcohols to follow the fixation in order to ensure the best possible morphological preservation of specimens. It has been pointed out earlier (Epstein and Holt, 1958) that by making the digestions in 30 per cent ethanol, adherence to this recommended series has been closely maintained. It was of course, known from these earlier studies that both types of nuclease were fully active in alcoholic solutions (Epstein and Holt, 1958).

Turning to the results which have been obtained, certain points of interest regarding the fine structure of the adenovirus particle have been established.

Thus, after permanganate fixation extracellular adenovirus has been found to have a hexagonal profile when seen in methacrylate sections. This agrees well with a very recent report regarding the shape of the intracellular form of the agent prepared in a similar way (Peters, 1959), whilst findings resulting from newly introduced techniques (Brenner and Horne, 1959) have demonstrated the basis of the 6-sided outline by showing that the virus particle is an icosahedron (Horne, Brenner, Waterson and Wildy, 1959). Earlier observations regarding the possible shape of the virus made on air-dried specimens mounted whole (Tousimis and Hilleman, 1957 ; Valentine and Hopper, 1957) have therefore been confirmed, thus ruling out the criticism that in such material the shape might have been caused by distortion during the drying (Morgan and Rose, 1959).

All reports on sectioned adenovirus in the past have agreed in describing the particle as round or ellipsoid (Kjellén *et al.*, 1955 ; Morgan *et al.*, 1956 ; Harford *et al.*, 1956 ; Lagermalm *et al.*, 1957) but in none of this work was permanganate used as the fixative : where it has been used, here and in other recent studies (Epstein, 1959 ; Peters, 1959), the hexagonal shape has been clearly preserved, and the importance of this method of fixation for the electron microscopy of viruses demonstrated yet again.

As measured here, the size of the virus particle corresponds well with that noted in previous studies using methacrylate-embedded sectioned material (Kjellén *et al.*, 1955 ; Morgan *et al.*, 1956 ; Harford *et al.*, 1956 : Lagermalm *et al.*, 1957), and if allowance is made for flattening during drying, it also agrees with estimates made by examining whole mount preparations (Tousimis and Hilleman, 1957 ; Valentine and Hopper, 1957) ; in addition, the dimensions of the nucleoid show a similar conformity.

The finding of a threadlike element within the nucleoid (Fig. 2 and 3) is considered to be of some importance ; the fact that it has only been seen in the very thinnest sections is probably due to the obscuring effect of the amorphous material also observed in this region of the virus.

Another important question regarding the structure of the adenovirus concerns its surface configuration. Particles prepared and sectioned directly have usually appeared to possess a smooth exterior contrasting markedly with the elaborate surface structure shown recently by new surface staining methods (Horne *et al.*, 1959). Now it could be that the techniques used in the present work failed to reveal existing surface structure ; but it could equally well be that the particle is in fact smooth and that some procedure used during the surface staining (Brenner and Horne, 1959) might have been responsible for bringing out an underlying structural pattern. That this could indeed have happened is suggested by the finding of a beaded appearance in the outer zone of particles incubated in watery medium before embedding. For in such particles favourably orientated to the plane of sectioning the beading appears to correspond to surface units (Fig. 4) and seems to have been made visible as a result of osmotic or extraction influences ; such factors, or a drying effect, could likewise have come into play in Brenner and Horne's surface staining procedure (Brenner and Horne, 1959 ; Horne *et al.*, 1959). But whether or not the virus is in actuality smooth, and whatever the mechanism permitting surface units to be seen, the present findings confirm those of Brenner and Horne (Horne *et al.*, 1959) in making it clear that the outer zone or viroplasm of the adenovirus has a basic geometrical substructure.

The presence of a definite limiting membrane around the adenovirus seems doubtful in view of the findings where high magnifications were used on very thin sections (Fig. 3 and 4). It is more probable that the viroplasm extends to the surface without a covering layer of different composition, the line of slight surface density discernible in the thicker sections (Fig. 1) representing perhaps traces of contaminating material deposited during extraction or minor changes in the surface of the particle caused during preparation.

The features of fine structure of the adenovirus discussed above are valid as far as they go, but they suffer from the limitation of being strictly morphological in nature. However, they provide a point of reference for the important question of integrating structure with composition which the digestion experiments of the present investigation were intended to elucidate. In the earliest of these experiments (Table I, Expt 1) the failure of RNase to affect the virus nucleoid directly was taken to indicate that this zone might contain DNA, since it was known that the relatively small RNase molecule could certainly penetrate and act upon permanganate-fixed Rous virus (Epstein and Holt, 1958). In view of the theoretical difficulty of getting the relatively larger DNase molecule into the virus, the negative results obtained when this enzyme was applied directly (Table I, Expt 2*c* and *d*) were followed by an experiment in which preliminary treatment with pepsin was undertaken before the nuclease digestions (Table I, Expt 3); this too left the nucleoid unaffected.

Three possible reasons for this further failure were considered and provided the rationale for subsequent experiments. Firstly, that although DNA in the nucleoid had been broken down by the DNase, some other associated material, loosened but not removed by the specific nuclease digestion, might have been responsible for the undiminished density in this case; a final treatment with pepsin was therefore tried out to attack such material (Table I, Expt 4) but without success. Secondly, that fragmentation of the DNA had not been completed; treatment with DNase II was accordingly applied, since this enzyme has been reported (Davidson, 1957) to cause very extensive breakdown of DNA. The procedure, however, was unsuccessful. Lastly, that after being fixed for 1 hr. with permanganate the nucleic acid could no longer be digested by DNase; a 15 min. fixation time was substituted (Table I, Expt 6) but again without influencing the result.

All these negative findings made it seem possible that neither the pepsin nor the acidity of the medium in which it was applied had affected the viroplasm, so that the DNase might perhaps have been unable to penetrate the virus and act on the nucleoid.

As an alternative method for opening up the viroplasm, mildly alkaline buffer was tested, and when this was followed by appropriate nuclease digestion, clear cut results were obtained for the first time (Table I, Expt 8). Thus, particles treated with buffer at pH 9·0 and then incubated with either RNase or 30 per cent ethanol with magnesium showed no change in the electron density of their nucleoids (Fig. 6 and 7) whilst in marked contrast to this the nucleoids were digested from similarly treated particles incubated with DNase (Fig. 5). In view of the specificity of the nuclease digestion and the findings of the control incubations these results demonstrate unequivocally that the adenovirus contains a substantial amount of DNA localized in the nucleoid.

The results obtained by the application of acridine orange staining and

fluorescence microscopy support the findings of the electron microscopy experiments regarding the nature of the nucleic acid in the adenovirus and this additional support is of particular significance since it is of an unrelated kind. Although light microscopy cannot resolve individual virus particles, the overall fluorescence response obtained from the stained smears with and without the nuclease digestions reflects the collective response of such particles since it had previously been shown that the material of which the smears were made consisted of them (Epstein, 1959 ; Epstein and Powell, 1960). Now, when stained with acridine orange and examined in UVL, RNA-containing structures usually exhibit an orange-red fluorescence and DNA-containing structures a yellowish-green fluorescence (Bertalanffy and Bickis, 1956 ; Armstrong, 1956), these colour responses being specifically diagnostic if they are abolished by treatment with the appropriate nuclease before staining. Thus, the red fluorescence given by the smears when stained directly with acridine orange (Fig. 8) cannot be ascribed to the presence of RNA since incubation with RNase before staining did not affect it (Fig. 9). This type of non-specific fluorescence is not uncommon with acridine orange staining techniques and examples of it have already been encountered with material of the kind used here (Epstein and Holt, 1958).

When the virus was treated with alkaline buffer it was clearly significantly affected, since after subsequent staining with acridine orange it gave a green fluorescence for the first time (Fig. 10). This green fluorescence definitely confirms the presence of DNA in the adenovirus in view of the fact that it was abolished in alkaline-treated preparations incubated with DNase before staining (Fig. 11) yet remained totally unaffected by similar incubation in enzyme-free medium alone.

Whatever the change in the permanganate-fixed virus particles which incubation in an alkaline medium brought about, it both permitted penetration of the nucleoid by DNase (Table I, Expt 8*b*) and altered the fluorescence response after acridine orange staining from a non-specific red colour to the green characteristic of DNA. The most probable effect of the alkaline buffer would seem to have been a degradation of the outer protein coat or viroplasm and this fits well with the findings of those who have dissected viruses chemically by using alkali to remove the outer protein coat in the course of preparing infective nucleic acid (Fraenkel-Conrat and Williams, 1955 ; Schramm, Schumacher and Zillig, 1955).

Although earlier work has shown DNA in regions of infected cell nuclei containing much virus (Bloch *et al.*, 1957), the basis of this demonstration was such that it could not establish whether DNA was present in the adenovirus or whether the DNA demonstrated was merely host cell DNA accumulated around and between the virus particles. The experiments reported here have not only established that DNA is present in the adenovirus, but also the site of this material within the particle. It has not however been possible to determine whether the nucleic acid was associated with the amorphous component of the viral nucleoid or the filamentous, but on structural grounds the latter seems possible.

Besides leading to the above findings the present work has illustrated again the special usefulness in virus research of the electron microscopy of thin sections when coupled with ancillary techniques. It is true, of course, that exact information regarding the composition of a virus is most reliably obtained by chemical analysis, but for this complete purification is a pre-requisite which can in itself be extremely difficult or often impossible to attain. The present approach has

the advantage not only of being able to furnish information on composition where only partially purified material is available, but also of being able to relate composition to morphology. Analysis can show what a given virus contains as a whole ; by examining structures *in situ* the methods used here can investigate the nature of its various parts.

SUMMARY

Experiments designed to investigate the fine structure and composition of type 5 adenovirus are described.

Pellets were prepared from fluorocarbon-treated suspensions of the virus and samples from their virus-containing zones were taken for examination both in thin sections with the electron microscope, and by fluorescence microscopy after acridine orange staining. Specific nucleases, other digestive treatments and appropriate control media were applied to the virus material in many of the experiments.

The results show that the adenovirus particle has a hexagonal outline and contains both an amorphous and a filamentous component in its nucleoid. The surface of particles prepared and examined directly appeared smooth, but surface structure was detected when the virus was exposed to osmotic and extraction influences.

It has also been shown that the adenovirus contains a substantial amount of deoxyribonucleic acid and observations made with the electron microscope have demonstrated its localization in the nucleoid.

These findings are discussed and the general significance of the methods used are considered.

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EXPLANATION OF PLATES

FIG. 1–7.—Electron micrographs of sectioned adenovirus particles from the virus-containing regions of pellets made from fluorocarbon-treated suspensions of the virus.

FIG. 1.—The 60 mμ virus particles are hexagonal in profile, contain a central dense nucleoid surrounded by a less dense viroplasm, and appear to have a very fine outer limiting membrane. ×160,000.

FIG. 2.—An individual virus particle in greater detail. The hexagonal profile is well seen and the nucleoid seems to contain 2 elements, a diffuse dense material and a threadlike component rolled in a haphazard manner into a ball. A definite outer limiting membrane is barely discernible. ×375,000.

FIG. 3.—Very thin section showing the structure of the nucleoid. The threadlike component is here arranged in curving parallel array. ×375,000.

FIG. 4.—Particles showing some extraction effects after incubation in watery medium at 37° for 6 hr. (Table I, Expt. 5*a*). A regular beading of the viroplasm can be seen in those particles favourably orientated to the plane of sectioning (arrows). The nucleoids have retained their density. ×150,000.

FIG. 5.—Group of virus particles prepared after incubation in buffer at pH 9·0 for 2 hr. at 37° followed by a further 2 hr. incubation in DNase. The nucleoids have been removed from the particles which appear considerably swollen. ×100,000.

FIG. 6.—Virus particles incubated as those shown in Fig. 5 except that RNase was used in place of DNase. Although some swelling of the particles has occurred, their nucleoids have not been digested. ×100,000.

FIG. 7.—Control particles incubated as those shown in Fig. 5 and 6 but treated in the second period of incubation with enzyme-free medium alone. The nucleoids are intact and only slight swelling can be seen. ×100,000.

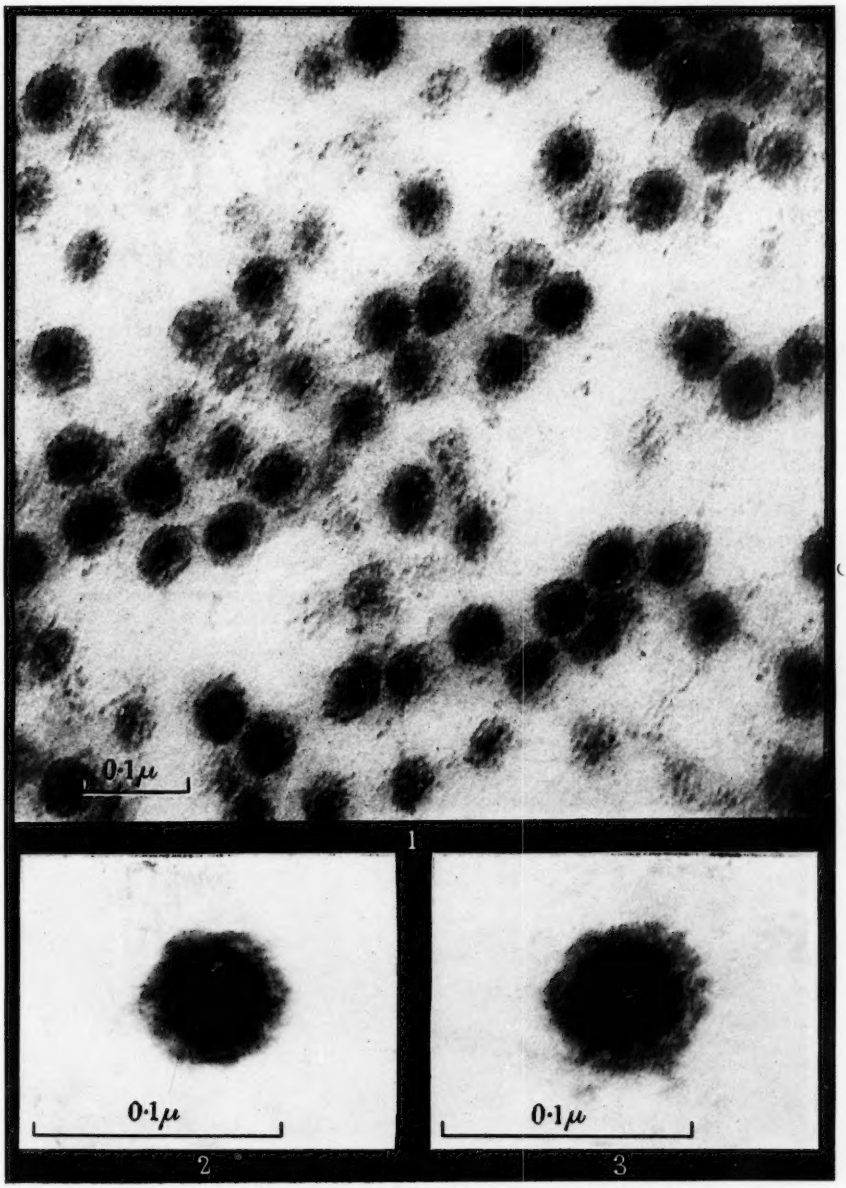
FIG. 8–11.—Fluorescence photomicrographs of acridine orange-stained smears from the virus-containing zones of pellets. ×160.

FIG. 8.—Orange red fluorescence of directly stained material.

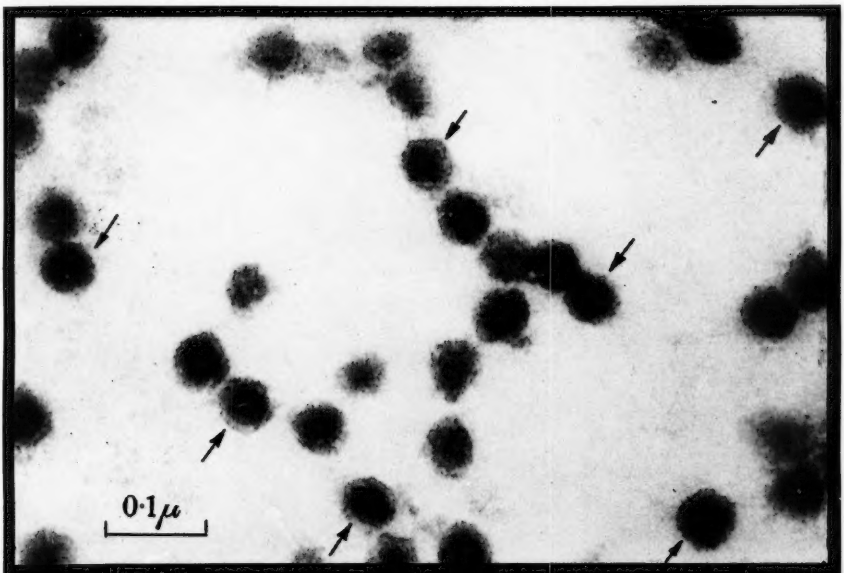
FIG. 9.—As Fig. 8 but stained after RNase digestion. Failure of the enzyme to change the colour of the fluorescence indicates that the latter is a nonspecific effect and not due to the presence of RNA.

FIG. 10.—Green fluorescence exhibited by material incubated in pH 9·0 buffer for 2 hr. and then stained with acridine orange. A few small crystals of the dye contaminate the lower right hand region of the background.

FIG. 11.—Feeble fluorescence given by a smear which had been incubated in pH 9·0 buffer for 2 hr., followed by a further 2 hr. incubation with DNase and then staining with acridine orange.



Epstein, Holt and Powell.



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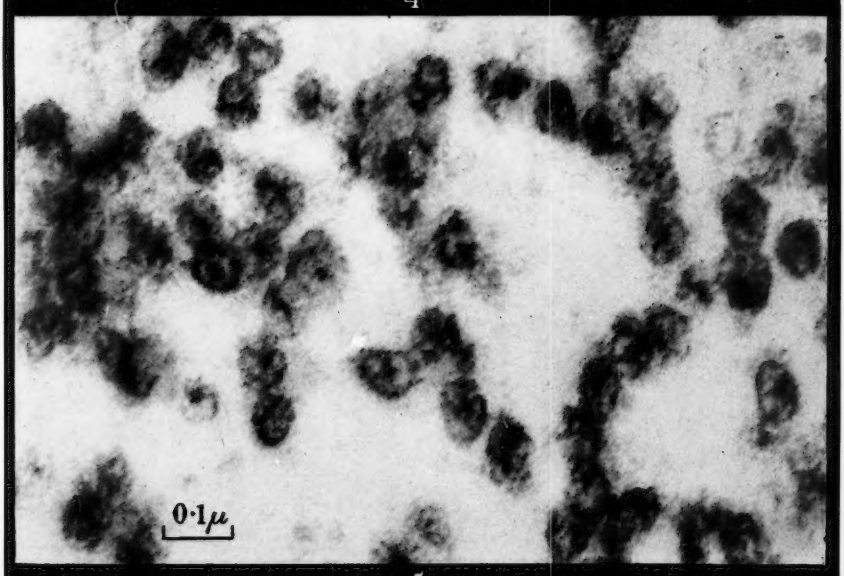

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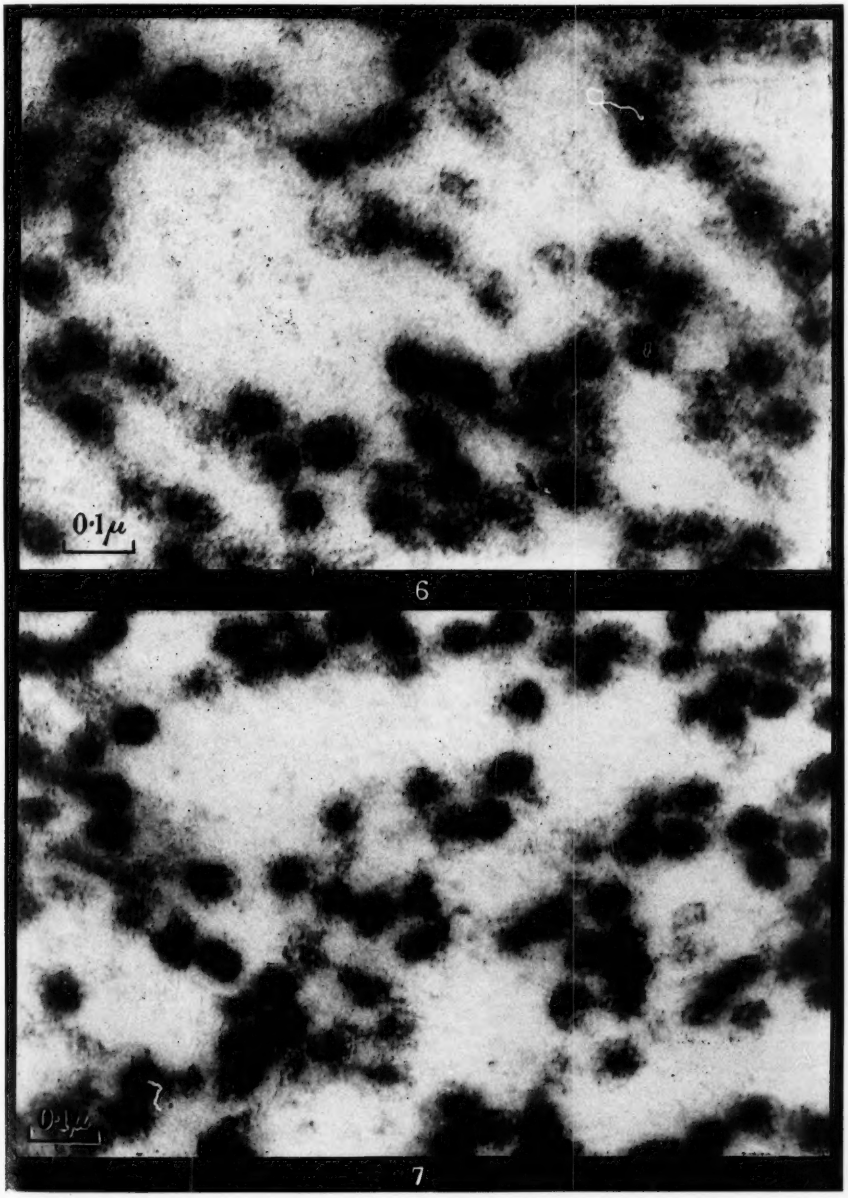

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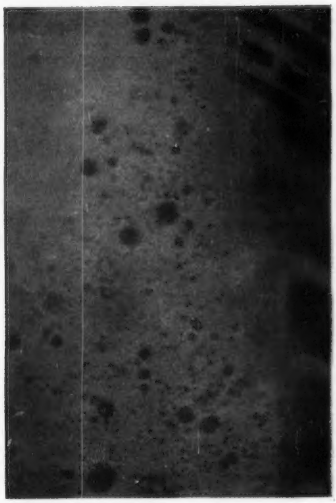

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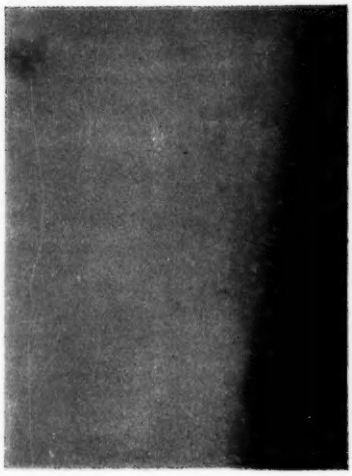

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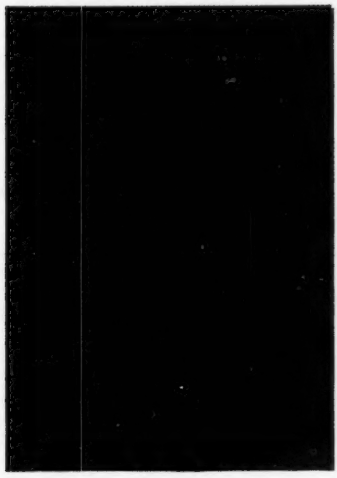

THE CHEMICAL BASIS OF THE VIRULENCE OF *PASTEURELLA PESTIS*

III. An Immunogenic Product Obtained from *Past. pestis* which Protects both Guinea-pigs and Mice

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One reason for examining *Past. pestis* (and its products) from infected guinea pigs was to identify the compounds responsible for the immunizing power of living avirulent *Past. pestis* for guinea pigs, because this was greater than the protection afforded by any of the products hitherto isolated from culture *in vitro* (Smith, Keppie, Cocking and Witt, 1960). In addition, an explanation was sought for the inability of Fraction I (Baker, Sumner, Foster, Meyer and Meyer, 1952)—the currently accepted basis for immunogenicity in mice—to account for the total immunizing activity of whole (dead) organisms for that species (Smith, *et al.*, 1960).

This paper describes the properties of an immunogenic product obtained from *Past. pestis* which immunized guinea pigs as well as mice. The product was first obtained from *Past. pestis* grown *in vivo*. Later a similar material was found in *Past. pestis* grown *in vitro* and experiments are described which prepare the way for the use of preparations containing this product in an improved non-living vaccine against plague. A preliminary report of some of the work has already appeared (Keppie, Cocking and Smith, 1958).

MATERIALS AND METHODS

Past. pestis (*strain L37*) *grown* in vivo

This was obtained as previously described, (Smith, *et al.*, 1960). A suspension of organisms (1×10^{10} per ml.) was used and all *Past. pestis* were killed by treatment with alkali as described later, or by heating to 60° for 1 hr. To provide other and possibly milder methods of killing whole *Past. pestis* for tests of immunogenicity in mice and guinea pigs (see Table I) the immunizing doses were made as follows: *Dose* 1. The suspension was injected with 0·02 mg./ml. streptomycin; guinea pigs and mice received 40 mg. and 3 mg. of streptomycin respectively on each of 4 successive days. *Dose* 2. Suspension treated for 24 hr. at 18° with hexyl resorcinol (0·0002 per cent w/v); tests in mice and on plates showed complete sterility of this material. Control animals (20) receiving doses 1 and 2 without the organisms, all succumbed to subsequent challenge in the active immunity test.

Extract (US) and Residue (R) obtained from Past. pestis *grown* in vivo *by treatment with ultrasonic waves.*—These were obtained as previously described (Smith *et al.*, 1960) and used at initial concentrations (approx. 3 mg. per ml. for US and approx. 1 mg. per ml. for R) equivalent to 1×10^{10} *Past. pestis* per ml. US was filtered sterile; if R was not dissolved in alkali it was sterilized by heating to 60° for 1 hr.

Residue (left after treatment with ultrasonic waves) from Past. pestis (*strains Tjiwidej and L37*) *grown* in vitro.—The medium of Davies (1956) was inoculated with *Past. pestis* strains Tjiwidej or L37 (1×10^8 per ml.) and shaken in Thompson bottles (250 ml. per bottle)

at 37° for 22–24 hr. Organisms were removed from the culture (7–8 l.; count 8–9 $\times$ 10^9 organisms per ml.) by centrifuging for 45 min. at 18,000 r.p.m. in the Spinco ultracentrifuge at 0–2°, and washed once with saline (1600 ml.). The bacterial deposit was suspended in distilled water (approx. 300 ml.) and aliquots (500 ml.) of the suspension (approx. 2 $\times$ 10^{11} *Past. pestis* per ml.) were treated with ultrasonic waves for 30 min. at 0–2° as previously described (Smith, *et al.*, 1960) for *Past. pestis* grown *in vivo*. The treated material (300 ml.) was diluted to 2 l. with distilled water and centrifuged at 10,000 **g.** for 45 min. at 0–4°.

The soluble material was removed and the residue washed once with distilled water (600 ml.) and finally suspended in sufficient saline (approx. 300 ml.) so that the suspension of residue was equivalent to 2 $\times$ 10^{11} *Past. pestis* per ml. before extraction with ultrasonic waves. The suspension was heated to 60° for 1 hr. to kill remaining *Past. pestis* and diluted to a concentration equivalent to 1 $\times$ 10^{10} *Past. pestis* per ml. (approx. 1 mg. dry wt. per ml.) as required for use.

Test for immunizing activity in guinea pigs and mice.—Two subcutaneous injections of the material under test were given to guinea pigs (450 g. $\pm$ 50) or to mice (20 g. $\pm$ 2) with an interval of two weeks between doses. One week after the final dose, virulent *Past. pestis* (strain L37) was given subcutaneously as a challenge; guinea pigs received 1 $\times$ 10^4 organisms (approx. 100 LD_{50}) and mice 1 $\times$ 10_3 (approx. 100 LD_{50}). The animals were observed for 21 days and the number of survivors noted.

In a few experiments (see Table IV) the virulent strains Shasta, M.P.6, 139L and M23 were used for challenge; we are indebted to Dr. T. W. Burrows for these strains.

Samples of Fraction 1.—Three samples were tested. Sample 1 (purified) and sample 2 (crude) were kindly supplied by Dr. K. F. Meyer (*via* our colleague Dr. Burrows). Sample 3—another purified specimen was produced by our colleague Dr. D. A. L. Davies.

Serological precipitation in gels.—The techniques and antisera used have been described (Smith *et al.*, 1960).

Analytical Methods

Ultracentrifugation (in Spinco Model L machine with a synthetic boundary cell) and electrophoresis (Spinco Series 10 machine).—We are indebted to our colleagues Mr. K. Cammack and Mr. K. Grinstead for these examinations.

Nitrogen.—By the Kjeldahl and Dumas methods.

Phosphorus.—By the method of Fiske and Subbarow (1925).

Glucosamine.—By a modification (Boas, 1953) of the Elson-Morgan method.

Lipopolysaccharide.—By the heptose reaction with H_2SO_4-cysteine (Dische, 1953) using the lipopolysaccharide of Davies (1956) as standard.

Total reducing sugars.—By the method of Somogyi (1952).

Sugars (calc. as glucose).—By the benzidine method of Pridham and Jones (1954).

Lipid.—This was weighed after extracting it from the material with chloroform/methanol as described by Weibull (1957) and purifying it by the method of Folch, Lees and Sloan-Stanley, (1954).

Nucleic acid.—By the extinction of light (260 mμ.) in a Unicam Spectrophotometer.

RESULTS

Products from Past. pestis *Grown* in vivo

Preliminary experiments showed that products of *Past. pestis* found extracellularly in exudates did not immunize mice and guinea pigs effectively. Hence attention was focussed on the organisms as a source of protective antigens.

Immunizing activity for guinea pigs and mice of the ultrasonic residue (R)

Active immunity tests with a suspension of R indicated clearly immunizing activity of this preparation for guinea pigs and mice (Line 1*a* Table I). Although R showed some toxicity in mice and guinea pigs when prepared under the conditions described in the previous paper, and injected intravenously (see Table I Cocking, Keppie, Witt and Smith, 1960), this toxicity was negligible under the conditions

of the test for immunizing activity, and in relation to the amounts of R shown to be active. Thus R $\equiv 0{\cdot}5 \times 10^{10}$ and 5×10^{10} organisms, did not kill any of 50 mice or guinea pigs respectively when injected subcutaneously.

TABLE I.—*Immunizing Activity of R Compared with US and Whole* Past. pestis *grown* in vivo

Material	Survivors / Number in group							
	Guinea-pigs					Mice		
	5*	1·0	0·33	0·1	0·03	0·1*	0·03	0·01	0·003
1. Ultrasonic residue (R)									
(*a*) Heated to 60° 1 hr.; insoluble	29/30	36/49	4/10	16/30	..	41/59	9/20	23/80	5/20
(*b*) Heated to 60° and then dissolved in alkali	..	22/25	18/22	16/25	..	47/90	..	7/40	..
(*c*) Unheated then dissolved in alkali	..	9/10	26/30	28/30	11/30	21/30	6/20	2/20	2/20
2. Ultrasonic extract (US)						Toxic			
(*a*) Filtered sterile . .	4/40	2/40	5/20	3/40	..	Toxic	6/9	18/19	13/20
(*b*) Treated with alkali as (*c*) above†	..	Not tested			..	Toxic 15/16	18/20	12/20	6/20
3. Whole organisms									
(*a*) Heated to 60° 1 hr. .	..	9/20	8/20	4/20	4/20	Toxic	Toxic 10/11	15/19	16/20
(*b*) Treated with alkali as in (*c*) above‡	..	15/20	11/20	10/20	7/20	18/19	15/20	10/20	9/20
(*c*) Treated with streptomycin and hexylresorcinol§	..	19/20	17/20	14/20	13/20	Toxic	Toxic 16/17	15/19	12/20

* Figures indicate the number of organisms ($\times 10^{10}$) or equivalent amount of product in each of 2 doses given s/c 2 weeks apaert.

† US treated with an equal volume of 0·2N NaOH for 5 min. at 18° followed by addition of glycine as described in text.

‡ Whole organisms treated as described in the text for R. Under these conditions approx. 20 per cent of the bacterial substance remained undissolved. The suspension was free from live *Past. pestis* and was not centrifuged and filtered before injection.

§ See methods.

Vertical lines indicate approx. concentration producing at least 50 per cent protection

Alkali used to dissolve R without loss of immunizing activity

R ($\equiv 40 \times 10^{10}$ *Past. pestis*) was mixed with 0·1 N NaOH (10 ml.) at 18°. After 5 min. solid glycine (approx. 450 mg.) was added until the pH was 9·2 and the final volume was adjusted with water to 40 ml. The slightly turbid solution was clarified by centrifugation (10,000 *g.* for 30 min. at 4°; insoluble material 3–4 per cent of R) and was shown by culture and the injection of mice to be free of living *Past. pestis.* Table I shows at line 1*b* the results of immunity tests with

R when it was dissolved in alkali after heating to 60° for 1 hr. and at line 1*c*, the results when heating was omitted. Comparison of these results with those in line 1*a* of Table I shows that the immunizing activity of R for guinea pigs was increased by dissoving it in alkali and the activity in mice was not significantly altered by this treatment. The immunizing activity of R for guinea pigs did not drop significantly even after treatment with alkali for several hours under these conditions.

For chemical and serological analysis a more concentrated solution (approx. 0·4 — 0·5 per cent w/v) of R was prepared by treating it ($\equiv 40 \times 10^{10}$ *Past. pestis*) with 0·1 N NaOH (10 ml.) as described above but not diluting with water after the addition of glycine.

Comparison of the immunizing activity of R with that of US and of whole Past. pestis

A comparison of the results of immunity tests in guinea pigs with R, US and sterile preparations of the whole organisms (Table I) showed that the activity of R largely accounted for that of the whole organisms and that US contained negligible activity. The toxicity for guinea pigs of US and the whole organisms (Cocking *et al.*, 1960) did not interfere with the immunity tests since no deaths occurred with either preparation during the immunization course of 2 subcutaneous injections spaced 2 weeks apart.

In mice, the US and the heated preparation of whole organisms were so toxic that immunizing activity could only be compared at low dosage. However, the toxicity of these preparations for mice could be substantially reduced by treatment with alkali (Cocking *et al.*, 1960) and this enabled the immunizing activity of the various preparations to be compared also at higher concentrations. The results in Table I show that although R had appreciable immunizing activity for mice, the activity of US was greater than that of R for this species. It is also evident (Table I) that treatment of whole organisms and US with alkali destroyed to some extent their immunizing activity for mice but not so rapidly as their toxicity for this species.

Properties of the immunogenic residue (R) obtained from Past. pestis *grown* in vivo

R was insoluble in water and saline at pH7 but could be dissolved by treatment with alkali as described previously and held in solution at pH 9·2 in 0·1 N NaOH–0·6 N glycine buffer. The material tended to come out of this solution as the pH was reduced and below pH7 it was precipitated.

Analysis.—Table II shows the analysis of a sample of the immunogenic complex prepared by dialysing its solution until free of salt and freeze drying; it contained protein, lipid and carbohydrate. Almost identical results were obtained by analysing R before dissolving it by treatment with alkali. Paper chromatography after acid hydrolysis of R indicated the presence of all the usual amino acids, glucosamine, glucose and the heptose which had been found in the polysaccharide of Davies (1956).

Ultracentrifugation and electrophoresis.—In the ultracentrifuge a small amount of R appeared to sediment rapidly to the bottom of the cell but the rest sedimented as a single peak (Fig. 1*a*) with $S = 4{\cdot}3$–$4{\cdot}7$ (assuming a $\bar{V}$ of 0·76); the peak spread rapidly indicating high polydispersity. On electrophoresis, R showed one major and one minor component (see Fig. 1*b*) with similar mobilities (the approx.

TABLE II.—*Analysis of Immunogenic Residue (R)*

Analysis	Per cent
N	10·9 (K), 11·7 (D)
Lipid	24·1
P	0·9
Ash	0·2
Glucosamine*	1·9
Reducing sugars (as glucose)†	2·8
Sugars detected by benzidine (as glucose)‡	1·8
Lipopolysaccharide	2·8
Nucleic acid derivatives	None detectable

Material dried *in vacuo* over P_2O_5 to constant weight.
K = Kjeldahl method. D = Dumas method.
* After hydrolysis with 6 N HCl for 3 hr. 100°.
† After hydrolysis with 6 N HCl for 18 hr. at 100°; corrected for reducing action of glucosamine. Similar results from a less drastic hydrolysis.
‡ After hydrolysis with 6 N HCl for 18 hr. at 100°.

mobility of the major component was $-6{\cdot}7 \times 10^{-5}$ cm./sec./volt/cm.). The behaviour of R in the ultracentrifuge and on electrophoresis was not significantly altered by heating it to 60° for 1 hr. before dissolving it in alkali, by prolonging the alkaline treatment to 17 hr., or by washing R with alkaline buffers prior to solution in 0·1 N NaOH.

Serological precipitation in gel diffusion plates.—A solution (0·4–0·5 per cent) of R in 0·1 N NaOH/0·6 N glycine buffer at pH 9·2 was prepared as described above. This concentrated solution formed 3 lines in serological precipitation plates against antiserum prepared against *Past. pestis* organisms (serum L.R.). One line was common with that formed by Fraction I and subsequent titrations indicated 0·2–0·4 per cent of Fraction I in R (assuming " free " and " combined " Fraction I reacted similarly). The other two lines (A and B) were common with 2 lines formed by a suspension of the lipopolysaccharide of Davies (1956). Against an antiserum (L.R.A.) adsorbed with avirulent organisms the solution of R formed one line, which was not common with those formed by the V and W antigens of Burrows and Bacon (1956).

An antiserum prepared in guinea pigs by injection of a solution of R formed two lines (corresponding with A and B above) when diffused against R but did not precipitate a solution of " free " Fraction I.

Immunogenic Residues from Past. pestis *Grown* in vitro

When the virulent L37 and the avirulent immunogenic Tjiwidej strains of *Past. pestis* were grown at 37° and immediately extracted with ultrasonic waves (see " Methods "), residues were obtained which, like the product obtained from *Past. pestis* grown *in vivo*, immunized both guinea pigs and mice (Table III).

Although somewhat less effective for protecting guinea pigs than R from *in vivo* organisms (cf. Table I line 1*a* with Table III) the residue obtained from Tjiwidej strain was investigated as the potential basis for a non-living vaccine against plague. The results shown in Table IV indicate that this residue protects mice and guinea pigs against challenge with several virulent strains of *Past. pestis* which were different from the L37 used routinely in this work.

TABLE III.—*Immunizing Activity for Guinea Pigs and Mice of Residues Left After Ultrasonic Extraction of a Virulent L37 and an Avirulent Immunogenic Tjiwidej Strain of* Past. pestis *Grown* in vitro

Strain from which residue† prepared	Survivors / Number in group					
	Guinea-pigs			Mice		
	5*	1	0·1	0·1*	0·03	0·01
Virulent L37 grown at 37°	8/10	13/15	7/15	23/30	..	10/40
Avirulent Tjiwidej grown at 37°	46/65	30/70	16/65	33/60	8/20	19/60

* Figures indicate the number of organisms ($\times 10^{10}$) from which the product in each of 2 doses (given s/c 2 weeks apart) was obtained.

† Prepared as described in " Materials and Methods " and injected as a suspension after heating to 60° for 1 hr.

Vertical lines indicate approx. dose which produced at least 50 per cent protection.

TABLE IV.—*Protection of Guinea Pigs and Mice Against Challenge From Several Virulent Strains of* Past. pestis *by the Immunogenic Product from* Past. pestis *(Strain Tjiwidej) Grown* In Vitro

Strain used for challenge†	Survivors / Number in group					
	Guinea-pigs			Mice		
	Nil	5·0*	1·0	*Nil*	0·2*	0·04
MP6	1/10	6/10	8/10	0/10	7/10	3/10
139L	0/10	10/10	6/10	1/10	6/10	7/10
Shasta	0/10	8/10	6/10	0/10	8/10	8/10
M 23	0/10	10/10	10/10	0/10	5/10	4/10

* Figures indicate the number of organisms ($\times 10^{10}$) or equivalent amount of product in each of 2 doses given s.c. 2 weeks apart.

† Numbers used for challenge were for guinea-pigs 7×10^5 organisms and for mice 1×10^3 organisms; otherwise the test for immunogenicity was the same as described in " Methods ".

Comparison of the immunogenicity of the products obtained from Past. pestis *grown* in vivo *and* in vitro *with that of Fraction I*

The results in Table V show that Fraction I injected without adjuvant had negligible immunizing activity in guinea pigs. and approx. 3–6 μg. was needed to protect 50 per cent of mice in the assay for immunizing activity. The corresponding protective dose for mice of 4 of our preparations (see Tables I and IV) are given below with their Fraction I content in brackets; the latter was estimated by serological precipitation in diffusion plates on the assumption that " free " and " combined " Fraction I react similarly. *Past. pestis* organisms grown *in vivo* 10 μg.

TABLE V.—*The Immunogenicity of* 3 *Samples of Fraction I for Mice and Guinea Pigs*

Sample	Survivors / Number injected							
	Guinea-pigs				Mice			
	120*	40	12	4 μg.	6	3	0·7	0·2 μg.
1 . .	4/20	8/20	4/20	2/19	19/20	8/20	8/20	2/20
2 . .	7/20	6/20	2/20	3/20	8/20	3/20	2/20	3/20
3 . .	3/20	2/18	3/20	1/20	12/20	14/20	6/20	4/20

* Figures indicate amount (μg.) injected s/c in each of 2 immunizing doses.

(2–3 per cent); R 100 μg. (0·4 per cent); US 10 μg. (2 per cent); residue from *Past. pestis* grown *in vitro*, 100 μg. (0·9 per cent). Hence the protective activity of all preparations for mice were much greater than that warranted by their content of Fraction I.

DISCUSSION

A product has now been obtained from *Past. pestis* which effectively immunizes guinea pigs, and accounts for the immunizing activity in this species of intact *Past. pestis* organisms isolated directly from infected guinea pigs. The product was present in the residue left after treating *Past. pestis* grown *in vivo* with ultrasonic waves. This residue also immunized mice against infection with *Past. pestis*, but was less active in this respect than the material dissolved by the action of ultrasonic waves; the latter did not immunize guinea pigs.

The immunizing activity of the residue or the extract for mice could not be explained by their estimated content of Fraction I. We have confirmed that the latter injected without adjuvant produced no significant protection of guinea pigs. None of our preparations induced immunological paralysis such as that reported by Spivack, Foster, Larson, Chen, Baker and Meyer (1958), after injecting large amounts of Fraction I into guinea pigs.

The residue, immunogenic for guinea pigs and mice, contained lipid, carbohydrate (including the lipopolysaccharide of Davies. 1956) and protein (including " combined " Fraction I) but no nucleic acid. We suggest that this residue is an important part of a cell wall complex of *Past. pestis* to which Fraction I (and possibly the virulence antigens) are attached firmly near the cell wall and loosely in the body of the envelope. This cell wall complex with the attached envelope can be regarded as the best antigenic material for the protection of all species. If it is split by autolysis, acetone drying, and to a lesser extent by washing or treatment with ultrasonic waves, then the fragments lose protective power. This loss may be more apparent in some species than others. Thus, the residue from ultrasonic treatment contains a relatively large fragment which retains high immunogenicity for guinea pigs but only to a low degree for mice. The extract contains smaller fragments—ineffective for guinea pigs—but collectively more effective for immunizing mice than one of them, namely Fraction I. In fact, it appears that Fraction I is an ill-defined fragment of a more complete antigen. Thus, whole organisms

and various products therefrom have greater immunizing activity for mice than is warranted by their content of Fraction I. Furthermore this antigen was originally isolated in 2 forms, fraction IA and IB ; IA, but not IB, contained carbohydrate and if anything IA had a greater immunizing activity for mice than IB (Baker *et al.*, 1952).

It is not yet known whether the residue left after extraction of *Past. pestis* with ultrasonic waves is a single complex since it has only been examined in solution after treatment with alkali. This treatment did not destroy the immunogenicity for guinea pigs but it was relatively drastic and may have hydrolysed some linkages. However, the solution of the residues thus obtained revealed no gross heterogeneity in the ultracentrifuge, and showed only 2 components in the electrophoresis apparatus. On serological precipitation in gel diffusion plates, against unabsorbed rabbit antiserum to live *Past. pestis*, a concentrated solution of the residue formed only 3 lines in contrast to the multiplicity of lines formed by the soluble extract or whole organisms.

It appears at present, that the compounds responsible for immunogenicity in guinea pigs are not the same as those responsible for toxicity. Thus, the residue did not contain as much of the toxicity of the original organisms, as did the extract (Cocking *et al.*, 1960), which however failed to protect guinea pigs.

The results with products from organisms grown *in vivo* suggested that the development of an efficient non-toxic, sterile, vaccine was possible if corresponding immunogenic products could be obtained from *Past. pestis* grown *in vitro*. This now having been achieved, the next obvious step will be to experiment with alkali treated residue, combinations of it with alkali treated extract, and with alkali extracted whole organisms. A vaccine may thus be obtained which is effective in immunizing guinea pigs, mice and maybe man.

SUMMARY

A product has been obtained from *Past. pestis* which immunized guinea pigs and accounted for the immunizing activity in this species of killed *Past. pestis* isolated directly from infected guinea pigs.

The product was a relatively non-toxic residue—cell-wall material containing lipid, carbohydrate and protein—left after extraction of *Past. pestis* with ultrasonic waves.

The residue also immunized mice but was less active in this respect than the extract ; the latter did not immunize guinea pigs.

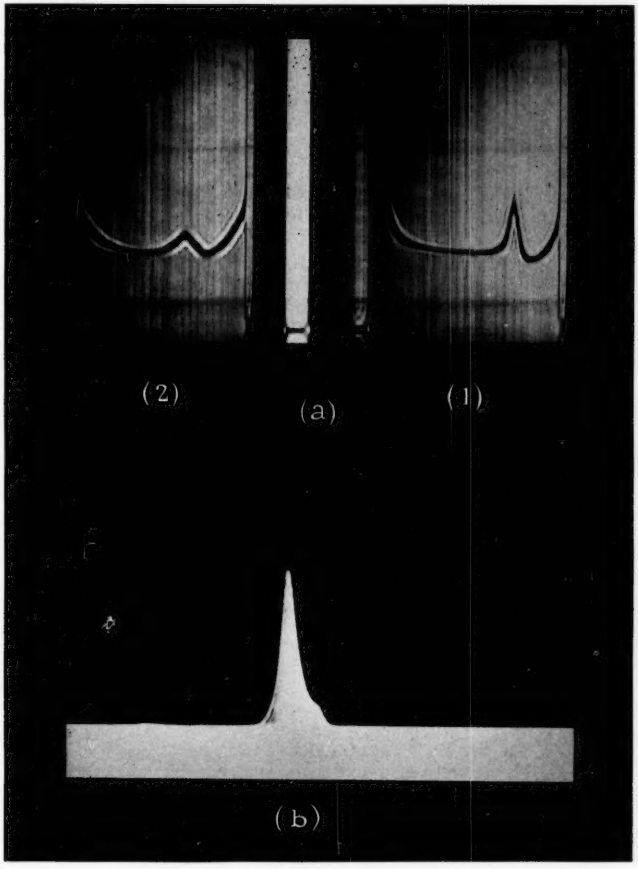
The immunizing activities for mice and guinea pigs of both residue and extract could not be satisfactorily explained on the basis of their estimated content of Fraction I.

EXPLANATION OF PLATE

FIG. 1.—Ultracentrifuge and electrophoresis diagrams on the immunogenic complex.
R (0·4–0·5 per cent w/v) was examined in 0·1 N NaOH/0·6 N glycine buffer at pH 9·2.

(*a*) Ultracentrifuge diagrams after 2 (1) and 18 (2) min. at 100,000 g. in a synthetic boundary cell.

(*b*) Electrophoresis diagram after 1 hr. in a field strength of 4·73 volt/cm. and a current of 12 mA ; ascending limb ; migration from left to right (anode).


(2)
(a)
(1)
(b)

It is suggested that the immunogenic residue is a relatively large fragment of a native cell wall—envelope complex which may be the best immunogen for all species.

The immunogenic product, first recognized in *Past. pestis* grown *in vivo* was later demonstrated in 2 strains of *Past. pestis* grown *in vitro*.

Various approaches now suggest themselves for the development of a sterile, non-toxic, vaccine which might be effective in immunizing man.

Our thanks are due to Mr. R. Blake and to Mr. G. Wilkins for excellent technical assistance.

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AN ANALYSIS OF THE TOXICITY FOR MICE OF INFLUENZA VIRUS

I. Intracerebral Toxicity

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Standard (non-neuro-adapted) strains of influenza virus are known to kill mice by a so-called toxic effect when large amounts are injected intracerebrally (Henle and Henle, 1944, 1946). Schlesinger (1950) showed that these strains underwent a single incomplete cycle of growth in the mouse brain, as detected by the production of haemagglutinin and complement fixing antigen. Cairns (1951*a*) however, showed that there was also an increase in infectivity titre during the cycle of growth, and that adapted strains differed by continuing to grow after this period.

The exact site of virus multiplication in tissues can now be demonstrated by the fluorescent antibody technique, and the intracerebral toxicity of standard influenza virus strains has therefore been analysed and compared with the behaviour of a neuro-adapted strain, using this technique.

MATERIALS AND METHODS

Viruses.—The MEL, PR8, and NWS strains of influenza A virus were used as allantoic fluid preparations. Red cell eluates, and concentrates obtained by centrifugation, were occasionally used.

Receptor Destroying Enzyme (RDE) (kindly provided by G. L. Ada) was used in the form of a *Vibrio cholerae* filtrate concentrated by adsorption-elution (Ada and French, 1950), and the principal experiments were repeated and confirmed using a pure, crystalline preparation (Ada and French, 1959). When 500 U RDE (French and Ada, 1954) were injected intracerebrally there was no protection against large doses of standard strains of virus given 1, 24, or 48 hr. later, and at least 1000 U were needed to give some degree of protection. Mice often showed hyperirritability and tremor after very large injections of impure RDE, and a cellular infiltration of the ventricles was detectable histologically.

Titrations.—Infectivity titrations were made in bits of allantois-on-shell as described by Fazekas de St. Groth and White (1958).

Haemagglutinin titrations were carried out in perspex trays, each cup containing 0·25 ml.; 0·05 ml. of 5 per cent fowl red cells were added, and partial agglutination taken as the end-point.

Mice.—Multicoloured outbred mice of the Walter and Eliza Hall Institute strain were used.

Fluorescent antibody technique.—Materials for examination were quick-frozen in tubes in a dry ice-alcohol bath, and thin sections cut on a Cambridge Rocker microtome in a −20° refrigerator (Louis, 1957).

Serum from a chicken hyperimmune to the MEL strain of influenza was used to prepare a fluorescent conjugate. A crude γ-globulin fraction of the serum was conjugated with fluores-

cein isothiocyanate (Sylvana Chemical Corporation, Orange, New Jersey, U.S.A.), and fixation, staining, washing and mounting of sections carried out in the usual way.

Sections were examined under a Zeiss microscope equipped for fluorescent observations, with an Osram HBO 200 mercury vapour lamp as light source. Photographs were made on HP3 (Ilford) film.

RESULTS

Standard strains (MEL and PR8)

In a preliminary experiment tenfold dilutions of the MEL and PR8 strains were injected intracerebrally and general features of the toxic effect observed. Deaths occurred within the first 6–7 days, and sick mice were hunched and ruffled, trembled, and often died in the course of tonic flexor or extensor convulsions (Henle and Henle, 1944). Such convulsions could be induced by tail twirling and sometimes merely by holding mice upside down. Doses of less than about $10^{6.0}$ ID_{50} (about 1 AD) were not usually lethal, and mice given doses slightly less than $10^{6.0}$ ID_{50} sometimes became sick and then recovered. Mice were well for a short time after the injection of a toxic dose of virus, and although with the largest doses sickness was detectable within 2 hr, there were no deaths until at least 12 hr. The same lethal endpoint was obtained whether toxic preparations were titrated in 1–7 day old or in adult (35–45 day) mice. There is therefore no great difference in the susceptibility of suckling and adult mice.

Brains were removed and examined by fluorescent antibody staining at intervals after the intracerebral injection of large amounts of MEL and PR8 virus, with the following results: 5 min.—marked fluorescence of cytoplasm of many ependymal cells and also of cells lining subarachnoid spaces. 30 min.—considerable fading of fluorescence. 3 hr.—almost no specific fluorescence detectable. 5–6 hr.—moderate fluorescence of cytoplasm of most ependymal cells. Faint fluorescence of cytoplasm of many of cells lining subarachnoid spaces. 7 hr.—fluorescence brighter. 8 hr.—further increase in brightness. Ependymal cell fluorescence much brighter than that of cells lining subarachnoid spaces.

There was no increase in the extent or brightness of fluorescence after 8 hr., and subependymal and subpial cells did not become fluorescent (Fig. 1).

Occasional fluorescent cells were seen in the Virchow Robin spaces as well as in the subarachnoid spaces. As a rule the ependymal cells covering the choroid plexuses showed a more intensive fluorescence than those in the ventricle wall, and there was often a brightly fluorescent cytoplasmic rim on the ventricular border of these cells. Most, but not all ependymal cells were fluorescent, and the few non-fluorescent cells were in groups involving, for instance, one side of a lateral ventricle.

The fact that there was no increase in the extent of fluorescence after 8 hr. suggested that there was a single cycle of growth. Most of the ependymal and subarachnoid cells, however, were fluorescent at this time after large doses of virus, and it was uncertain whether growth was limited in this experiment because only these cells were capable of supporting growth, or because growth was restricted to a single cycle. Accordingly 10- and 1000-fold dilutions of stock material were injected intracerebrally, and the brains were examined after 12, 17, 24 and 36 hr. A smaller proportion of the ependymal and meningeal cells became fluorescent with these doses, and there was no increase in the extent of fluorescence after 12 hr. Growth therefore is restricted to a single cycle. In this experiment ependymal cells were still brightly fluorescent at 4 days, although the fluorescence was

in denser, better defined cytoplasmic aggregates. By 7 days fluorescent cells were no longer visible.

A neuro-adapted strain (NWS)

The NWS allantoic fluid preparations used were lethal when diluted 10^{-5} (to contain about 1/1000 A.D.) before intracerebral injection. Deaths occurred from the 4th–7th day, depending on the dose, and although fatal convulsions occurred and could be induced by tail twirling they were less common than with unadapted strains of virus.

In a number of experiments undiluted allantoic fluid was injected intracerebrally and brains removed at different times and examined by fluorescent antibody staining (Fig. 2–5). By 8 hr. there were a moderate number of fluorescent ependymal cells, which were brighter and more numerous by 12 hr. At this time the fluorescence was identical in distribution to that seen with unadapted strains. By 24 hr., however, there were fluorescent cells extending away from the ependyma into the parenchyma. These extensions into the parenchyma were well marked by 36 hr., and even more pronounced at 48 hr. Collections of fluorescent cells were also seen below the meninges. It was not possible to decide whether the fluorescent parenchymal cells were glial or nerve cells.

NWS thus differs from unadapted strains in its ability to invade the parenchyma from the ependyma and meninges. In another experiment where smaller doses of NWS were injected and brains examined at 12, 24, 36, 60, and 83 hr., distinct increases in the extent of ependymal fluorescence were observed. This means that NWS is capable of successive cycles of growth in the ependymal sheet of cells.

Histological appearances

Henle and Henle (1946) reported that toxic doses of unadapted influenza virus strains produced destruction of the ependymal lining of the ventricles. This could be confirmed in sections of brain stained with haematoxylin and eosin. In addition there were large numbers of inflammatory cells in the ventricles, together with some blood, and there was an infiltration of cells into neighbouring areas of parenchyma. Choroid plexuses were often reduced to a few necrotic fragments. Ventricular profiles sometimes showed a slight hydrocephalic bulge and contained large amounts of fluid, as well as cells. In mice dying after 6–7 days the hydrocephalic tendency was more marked and the cellular reaction was more pronounced, for ventricles were often filled to capacity with inflammatory cells, and a more extensive infiltration of the parenchyma was seen.

The severe inflammatory response, originating in the ventricles, might result in an increase in intracranial pressure, as well as interfere with the production and circulation of cerebrospinal fluid. The signs and symptoms of cerebral oedema can sometimes be relieved by intravenous injections of hypertonic glucose, which reduce the intracranial pressure. The intraperitoneal or intravenous injection of 50 per cent glucose, however, had no detectable effect on the sickness of mice who had received toxic intracerebral injections of influenza virus.

Kitayama, Sunakawa and Fukumi (1953) showed that in NWS infected brains the principal pathological changes were in ependymal cells, and these are the cells which have been shown above to be primarily involved following intracerebral injections, even of small doses of virus.

Effect of RDE

Wagner (1952) reported that large intracerebral doses of RDE gave complete protection from the toxic effect of maximal intracerebral doses of standard influenza virus strains. This was readily confirmed, whether RDE was injected 1 hr. or 48 hr. before virus. When, however, brains of RDE-protected mice were examined by fluorescent antibody staining 24 hr. after a large intracerebral virus injection it was found that there had been growth in occasional ependymal and meningeal cells, although far fewer than in control mice. Protection by RDE was thus not absolute, at the histological level. In these experiments RDE and virus were injected into the same hemisphere, and although the injected material floods through the CSF spaces (Mims, 1960) it might not reach all the cells lining these spaces. It was pointed out earlier that even with the largest virus doses given to normal mice, there were occasional non-fluorescent ependymal regions at the time of death. Thus, virus might grow in ependymal and meningeal cells which had not received enough RDE as a result of slight differences in the distribution of virus and RDE.

When 1000-fold diluted stock virus was injected intracerebrally into RDE-protected mice fluorescent cells could not be detected at 24 hr. whereas there were moderate numbers of fluorescent ependymal cells in control non-protected mice. RDE therefore gives more effective protection against smaller doses of virus.

Cairns (1951*b*) showed that RDE treated mice were protected against moderate doses (up to 100 IC LD_{50}) of the NWS strain of virus ; this could readily be confirmed. It was shown in fluorescent antibody experiments that, as with standard strains of virus, protection against large doses of virus was not absolute at the histological level, for there were a number of fluorescent ependymal and meningeal cells 24 hr. after an RDE treated mouse had received a large intracerebral injection of NWS virus. These initially infected cells presumably infect neighbouring but unprotected cells, and a progressive spread of the infection through unprotected cells kills the mouse in the usual way.

The response of suckling mice

When 1–3 day old mice were injected intracerebrally with large doses of the MEL and PR8 strains they died within 24 hr. The heads were sectioned and stained with fluorescent antibody. The skin was removed from the heads because the stratum corneum has a brilliant yellow fluorescence, fragments of which tend to be dragged across the section by the microtome knife. Specific fluorescence was confined to the meninges and the ependyma, although occasionally more than one layer of cells were involved in ependymal regions (Figs. 6 and 7). Smaller doses of virus were then injected so that mice died after 3 or more days, and heads were examined at 12, 22, 27 and 46 hr. If mice were injected when less than 24 hr. old, it was clear that there was an increase in the number of fluorescent cells after 12 hr., and up to 6 layers of subependymal cells were often involved. There was less evidence for an increase in fluorescence in older suckling mice. In these sections brightly fluorescent cells lining the oronasal passages were often seen (Fig. 8), and this demonstration that there is growth of intracerebrally injected virus in these cells confirms the findings of Tyrrell and Cameron (1957), who described pathological changes in the nasal mucosa of suckling mice infected in this way. India ink was injected intracerebrally to infant mice, whose heads were

immediately removed, and haematoxylin and eosin stained sections prepared. Ink was present in submucosal lymphatics, in a few veins, and in the submucosal connective tissue spaces in some areas. Intracerebrally injected virus would be distributed to the submucosa in the same way and would therefore be able to grow in mucosal cells. There was a similar distribution of India ink in intracerebrally injected adult mice. The fluorescent cells lining the nasal passages were commoner when mice survived more than 2 days, and from the distribution of these cells it was clear that more than one cycle of growth was involved.

Thus, standard strains grow in the ependymal and meningeal cells of suckling mice, and at least in mice injected when less than 24 hr. old there are successive cycles of growth. The injected material also reaches the nasal mucosa, and growth here is not restricted to one cycle.

Similar experiments were done with the NWS strain, and there was extensive involvement of the brain parenchyma after preliminary growth in the meninges and parenchyma, as in adults.

DISCUSSION

Although the greater part of an intracerebral inoculum immediately spills over into the blood (Cairns, 1950), that part of the inoculum which stays in the brain is deposited in the cerebrospinal fluid spaces (Mims, 1960). The cells lining these spaces, therefore, are directly exposed to the injected virus, and may support virus growth. In the case of poxviruses growth occurs principally in these cells while there is a limited extension of the infection to the brain parenchyma (Mims, 1960). This extension into the parenchyma is perhaps a result of the migration of infected subependymal cells, for these cells are known to proliferate and then migrate into the parenchyma in the adult mouse (Messier, Lebland and Smart, 1958).

Both standard and neuro-adapted strains of influenza virus, evidently, grow in the ependymal and meningeal cells immediately available to them after intracerebral injections. Standard strains are only capable of a single cycle of growth, so that there is no spread of the infection to underlying cells, nor to neighbouring uninfected cells. If enough virus is injected there is a lethal amount of growth in this single cycle, and the so-called toxic effect is produced. At least 10^6 ID_{50} have to be injected to produce this effect, and if it is assumed that at the most 10 per cent of the inoculum stays in the brain, then 10^5 ID_{50} infect enough cells to kill the mouse.

In spite of the fact that there is no further growth of virus after about 8 hr., mice often survive for several days. Mice probably die as a result of the cellular reaction to infection, rather than to the mere growth of virus in ependymal cells, although a disturbance in cerebrospinal fluid production must result from the heavy infection of the ependymal cells covering the choroid plexuses. Perhaps one way in which the intense cellular reaction contributes to death is by obstructing the flow of cerebrospinal fluid, eventually producing the frankly hydrocephalic condition seen in mice dying after 6–7 days.

As was pointed out by Cairns (1954) the inability of standard strains to undergo more than one cycle of growth might be due to a defect in the virus produced in this cycle, to its retention in the cell, or to some other inability to spread to other cells. At least part of the virus produced is infective for the egg (Cairns, 1951*a*), although non-infective virus is produced when large inocula are given (Schlesinger,

1950), and it may be that there is an inability to spread to other cells. The non-infectious haemagglutinin produced by influenza virus in HeLa cells is retained in cells until they are destroyed (Henle, Girardi and Henle, 1955). The fact that antigen often accumulates at the ventricular edge of ependymal cells suggests defective release, but similar appearances were seen by Watson and Coons (1954) in infected cells of the chick embryo. It is known that fowl plague antigen accumulates at the cell membrane before virus is released (Breitenfeld and Schäfer, 1957).

Standard strains behave differently when injected into mice less than 24 hr. old for there are then successive cycles of growth in the brain.

Neuro-adapted strains of influenza virus undergo a primary period of growth in the ependymal and meningeal cells immediately available, and their adaptedness lies in their ability to undergo successive cycles of multiplication, for they then invade underlying parenchymatous tissues, as well as neighbouring uninfected cells. There is a similar spread of infection in infant mouse brain. Whether virus grows in neurones or only in neuroglial cells, death might result from the damage to these parenchymal cells. Alternatively after there has been a lethal extent of growth in the ependyma and meninges, death could occur in the same way as with unadapted strains.

The results of RDE pretreatment are understandable in the light of the different growth patterns of standard and neuro-adapted strains. It was shown that RDE protection against large doses of virus is not absolute at the histological level, perhaps because of minor differences in the distribution of intracerebrally injected RDE and virus. Most ependymal and meningeal cells, however, are protected, whether virus is given 1 hr. or 48 hr. after RDE. Standard strains, which are lethal only if they grow in most of these cells, are then no longer lethal. The neuro-adapted strain, on the other hand, since it also can infect a few of these cells and is capable of successive cycles of growth, is still lethal. The lethal endpoint is reduced about a hundredfold (Cairns, 1951*b*), because with small doses of virus RDE protection is complete.

A similar investigation of the growth of intracerebrally injected influenza viruses has recently been made by Fraser, Nairn, McEntegart and Chadwick (1959). Their findings are substantially the same as those reported here.

SUMMARY

Standard strains of influenza virus undergo a single cycle of multiplication in the ependymal and meningeal cells immediately available to them after intracerebral injection. If enough cells support virus growth mice die and the so-called toxic effect is produced. RDE prevents the infection of most cells and thereby protects against toxicity.

Standard strains undergo successive cycles of growth when injected into the brains of mice less than 24 hr. old. Some of the injected virus is deposited below the nasal mucosa, and there is then growth in mucosal cells.

Neuro-adapted strains of influenza virus, which undergo successive cycles of multiplication in adult mice, grow first in ependymal and meningeal cells, and then in underlying parenchymal cells. Although RDE prevents the infection of most ependymal and meningeal cells a small number of these cells support virus growth, which then slowly progresses, and may kill the mouse.

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EXPLANATION OF PLATES

FIG. 1.—Fluorescent antibody stained section of adult mouse brain 17 hr. after the intracerebral injection of a large dose of MEL virus. Ventricular profile with fluorescence limited to ependymal cells. ×124.

FIG. 2.—Fluorescent antibody stained section of adult mouse brain 17 hr. after the intracerebral injection of a moderate dose of NWS virus. Ventricular profile with fluorescent ependymal cells limited to the tips of the choroid plexuses. ×93.

FIG. 3.—Fluorescent antibody stained section of adult mouse brain 36 hr. after the intracerebral injection of NWS virus. Fluorescence is beginning to extend from the ependymal to the subependymal cells. × 93.

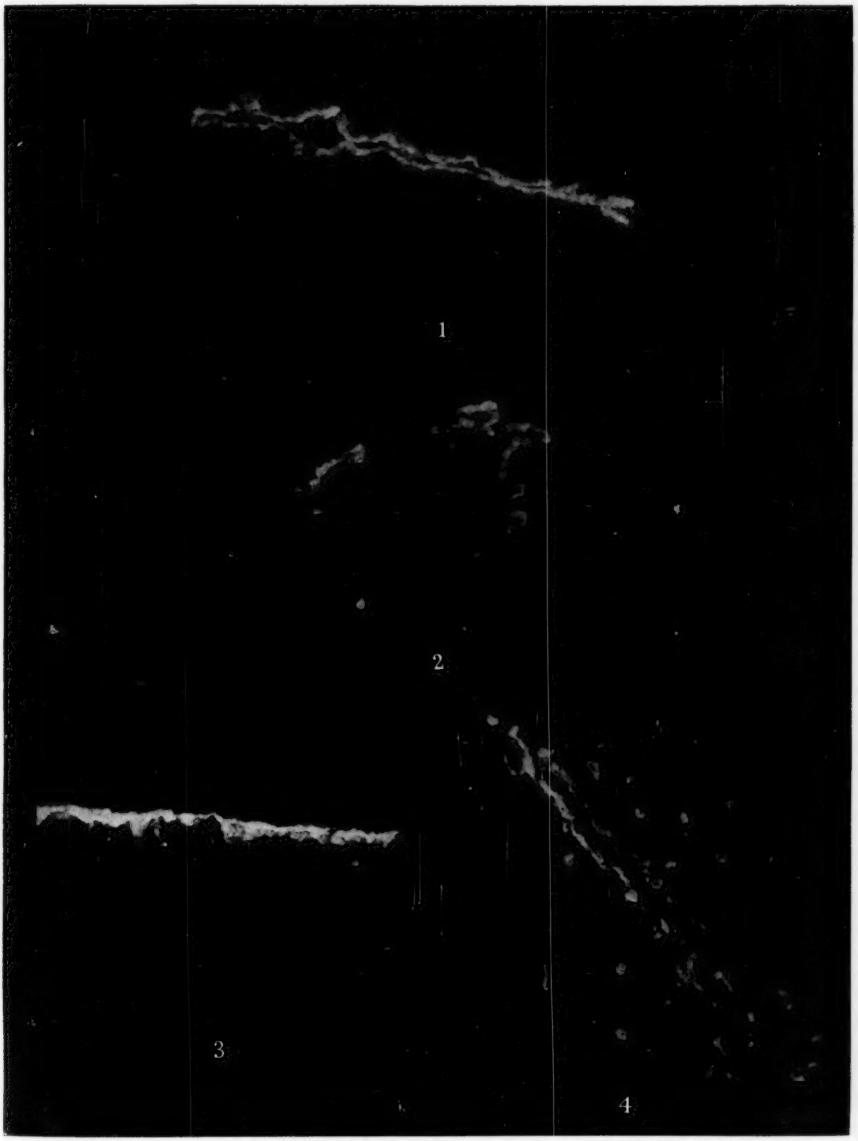
FIG. 4.—Fluorescent antibody stained section of adult mouse brain 3½ days after the intracerebral injection of NWS virus. A sulcus, with fluorescent cells lining the subarachnoid spaces, and numerous fluorescent cells in the neighbouring parenchyma. ×124.

FIG. 5.—The same section (NWS 3½ days), showing fluorescent parenchymal cells. ×243.

FIG. 6.—Fluorescent antibody stained section of suckling mouse head 24 hr. after the intracerebral injection of the PR8 strain of influenza virus. Ventricular profile with fluorescent ependymal cells covering tips of choroid plexuses, and extension of fluorescence into subependymal cells. × 90.

FIG. 7.—Same slide showing fluorescent cells on meningeal surface of brain. × 60.

FIG. 8.—Fluorescent antibody stained section of infant mouse nasal mucosa 6 days after intracerebral injection of the PR8 strain of influenza virus. A focus of fluorescent mucosal cells. ×60.



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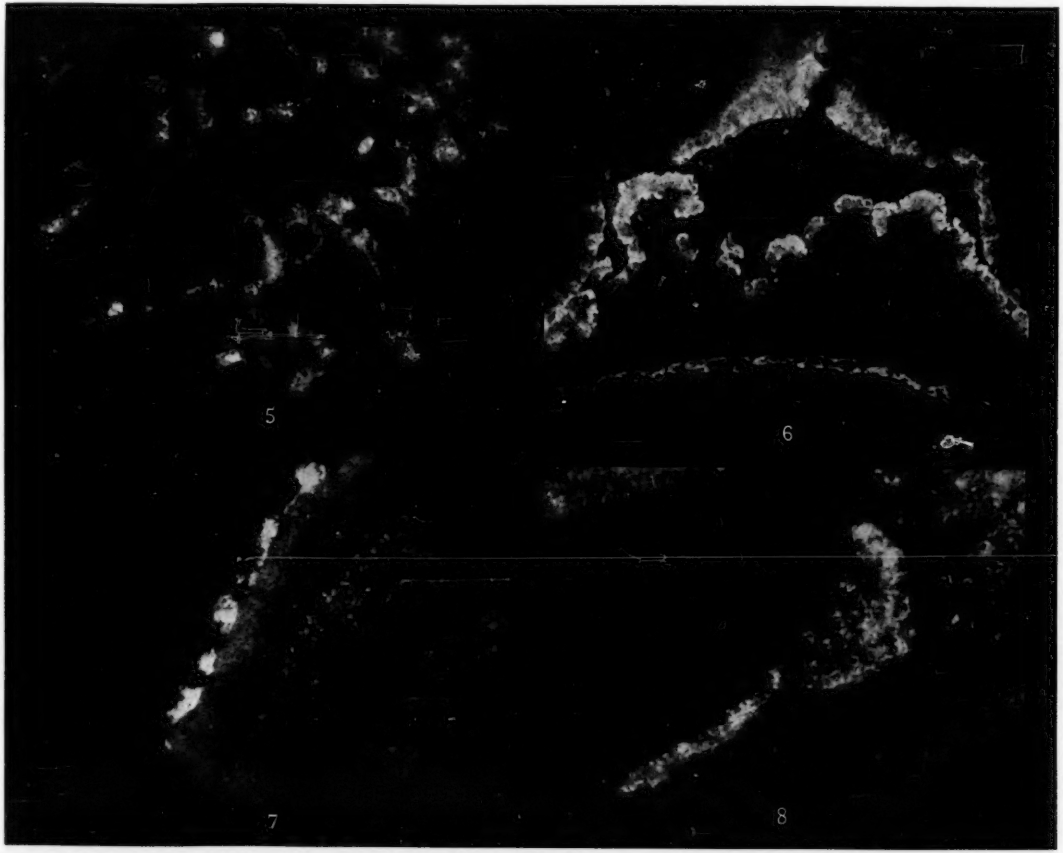

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AN ANALYSIS OF THE TOXICITY FOR MICE OF INFLUENZA VIRUS

II. Intravenous Toxicity

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Standard strains of influenza virus were shown by Henle and Henle (1946) to kill mice by a so-called toxic effect when massive doses were injected intravenously. Pathological changes were produced in the liver, spleen and gut, but they obtained no evidence that the injected virus multiplied in organs other than the lung. The toxic property was associated with the virus particle.

After the intracerebral toxicity of standard strains of influenza virus had been analysed, as described in the preceding paper, a similar investigation of intravenous toxicity was undertaken.

MATERIALS AND METHODS

Materials and methods are those previously described (Mims, 1960). The best toxic preparations were of the PR8 strain of influenza virus and this strain was used in nearly all experiments. The CL strain of vaccinia virus is a dermal strain (Fenner, 1958): CL-R is a neurovaccinia strain derived from it during passage in mouse brain (Woodroofe, unpublished).

EXPERIMENTAL RESULTS

Preliminary experiments

The toxic action of intravenously injected virus killed mice within 4 days. At the time of death the liver was enlarged, mottled red, and friable. The gall bladder was often haemorrhagic, and a haemorrhagic enteritis extended from the duodenum to the terminal ileum. A fresh peak of deaths was seen at 5–10 days, and, as Henle and Henle (1946) pointed out, these were due to the pulmonary consolidation resulting from the growth of virus in the lung. These late deaths occurred with about one-tenth as much virus as was needed for the toxic action. At the time of the toxic death, the lung appearances were very variable, for while there was occasionally fairly extensive consolidation, there was often no macroscopic abnormality.

The toxic effect was not produced unless $10^{8.5}$ ID_{50} of virus, with an infectivity-HA ratio of at least $10^{5.5}$, was injected. Ratios lower than this were repeatedly obtained from eggs at the time that this work was in progress, but such preparations were not toxic, even when as much as 20,000 AD of concentrated material was injected. The toxic action thus requires large doses of infectious virus, and does not occur if there is too high a proportion of non-infective material.

Growth curve experiment

Livers were removed from mice at various times after the intravenous injection of a toxic dose of virus. For each sample two livers were pooled, snap frozen at

−60° and later thawed, ground to a paste in a chilled mortar and suspended in gelatin saline (0·5 per cent gelatin in normal saline). Suspensions were dispersed by 20 sec. treatment with an ultrasonic disintegrator, and then centrifuged at 2000 *g* for 5 min. before titration. The result of this experiment (Table) shows that

TABLE.—*Log Infectivity and Haemagglutinin Titres per Liver at various Times After the Intravenous Injection of a Toxic Dose of PR8 Virus*

	Time						
	3 min.	3 hr.	6 hr.	7 hr.	8 hr.	12 hr.	18 hr.
Infectivity titre . .	7·7	4·5	4·2	3·2	4·3	5·1	5·6
Haemagglutinin titre .	3·7	<3·0	<3·0	<3·0	<3·0	3·0	3·9

after a considerable initial fall in infectivity titre, there was a smaller but distinct rise from the 8th hr. onwards. The 18 hr. livers were taken from 1 recently dead and 1 very sick mouse.

To test for the possible inactivation of virus by liver, a normal liver suspension was prepared in the way described above, and 0·05 ml. of this suspension added to each 1 ml. dilution in a standard influenza virus titration. Virus dilutions plus liver were incubated for 15 and 90 min. before testing for infectivity, but the liver caused no reduction in titre as compared with a control titration.

Haemagglutinin titrations of the same liver samples showed that there was also a rise in haemagglutinin titre from 8–18 hr. (Table). There was little or no haemagglutinin inhibition by normal liver suspensions when normal virus was used, although high inhibitory titres were detected with indicator virus.

It is concluded that there is a small although distinct rise in infectivity and HA titres after the 8th hr. In all the samples infectivity-HA ratios are exceedingly low, especially at 18 hr., and even at 3 min. This means, firstly, that nearly all the virus material produced is non-infective, and secondly, that the littoral liver cells which take up injected virus have rendered much of it non-infective within 3 min.

Fluorescent antibody and routine histological observations

Many mice were injected with a toxic dose of virus, and livers removed at various times and examined both by routine haematoxylin and eosin and by fluorescent antibody staining.

5 min.—fluorescence of most littoral cells lining sinusoids (Fig. 1). No fluorescence of hepatic cells. 3 hr.—very marked fading of fluorescence which is only detectable in occasional littoral cells. A notable increase in the number of mononuclear cells present in sinusoids, a few of them with pyknotic fragmented nuclei. 5 hr.—no change in appearances. 7 hr.—distinct increase in number of fluorescent littoral cells, which are now brighter. A smaller number of faintly, although distinctly fluorescent hepatic cells are present, fluorescence involving principally the peripheral and perinuclear regions of the cytoplasm. 8 hr.—fluorescent hepatic cells brighter with a rim of cytoplasm showing particularly bright fluorescence in many cases. Further increase in number of fluorescent littoral cells, and occasional collections of such cells in sinusoids. A number of fluorescent mononuclear cells in veins. Liver now enlarged, with a reddish, slightly mottled appearance. Not more than a few degenerate hepatic cells in haematoxylin and eosin sections. 18 hr. —many, but by no means all hepatic cells fluorescent and counts are difficult

because some cells are brightly and some only just detectably fluorescent. Fluorescent cells do not occur in groups (Fig. 2). Occasional fluorescent littoral cells. Liver greatly enlarged, mottled red, and friable. Gall bladder haemorrhagic. Haemorrhagic enteritis. Confluent pathological changes in liver in haematoxylin and eosin sections. Many hepatic cells have no nucleus, others have pyknotic nuclear fragments scattered throughout the cytoplasm, which is often vacuolated. Large numbers of cells, together with pyknotic nuclear debris, in sinusoids. Pyknotic nuclear debris and cell-fragments also present in veins.

An extensive production of antigen in the liver therefore accompanies the toxic action of intravenously injected influenza virus.

In another experiment subtoxic doses of virus were injected and livers again examined by fluorescent antibody staining. It was found that when one-quarter of the minimal toxic dose was given, there were few if any fluorescent hepatic cells visible after 18 hr. With one-tenth of the minimal toxic dose there was no increase in littoral cell fluorescence by 12 hr., and it is concluded that virus grows in these cells only when near-toxic doses are given. Occasionally with large doses of non-toxic virus stocks of a low infectivity-HA ratio, there were significant increases in littoral cell fluorescence at 8 hr., although hepatic cells did not become fluorescent. Toxicity, therefore, is closely associated with the production of fluorescence in hepatic cells.

Scattered collections of fluorescent cells were seen in the red pulp of the spleen, and very occasional fluorescent cells in the lung, duodenum, small intestine, and kidney. Thus nearly all the antigen produced is produced in the liver.

Bile duct injections

In other experiments it was shown that subtoxic but nevertheless very large intravenous injections of virus were taken up by the littoral cells lining the liver sinuses. Antigen then faded in these cells, and usually failed to reappear. With the slightly larger but toxic doses, however, antigen was produced both in littoral and in hepatic cells, as was described above. The littoral cells therefore can deal successfully with very large amounts of virus, but when they take up more than a certain amount they allow virus to " leak " through to hepatic cells and at about the same dose level they themselves support virus growth. It seemed of interest to test the susceptibility of hepatic cells to influenza virus, independently of the protective barrier of littoral cells.

In an attempt to introduce material into hepatic cells by way of the biliary channels, 0·2 ml. of a 1 : 5 dilution of India ink in saline was slowly injected into the common bile duct of an anaesthetized mouse. The liver was removed a minute later, fixed in formol saline, and haematoxylin and eosin stained sections prepared. There were groups of hepatic cells, usually round the portal tracts, whose cytoplasm contained varying amounts of India ink (Fig. 3). The bile ducts themselves were usually free of India ink, but the lymphatic channels in the portal tracts contained considerable amounts, often partly enclosing the portal veins. Ink was also present in many littoral cells, although some of it may have been in the spaces of Disse. It was concluded that as the pressure in the biliary tracts rises during the injection, anatomical barriers are broken down at the site of periportal hepatic cells. The injected material flows through some of these cells into the spaces of Disse, and thus enters the lymphatic channels. India ink was often present in veins and there was evidence, in the form of broken down walls of veins adjacent to the bile

ducts, that there was a spill over from the bile ducts directly into the blood. India ink may have entered littoral cells from the spaces of Disse as well as from the blood. Material injected into the common bile duct in this way, therefore, is introduced directly into the cytoplasm of many hepatic cells.

In a number of experiments, virus preparations coloured with 1 per cent Trypan blue were injected into the common bile duct, the laparotomy wound sutured, and mice killed and livers examined at different times afterwards. At 2 hr. after the injection there were no specifically fluorescent cells, although there was already a moderate mononuclear cell infiltration of the portal tracts. By 8 hr. there were many brightly fluorescent hepatic cells, usually round the portal tracts, with the fluorescence often concentrated in the perinuclear and peripheral regions of the cytoplasm as after intravenous injections. The cells lining bile ducts were occasionally fluorescent. The fluorescent hepatic cells were brighter at 18 hr. (Fig. 4 and 5) but even after 30 hr. they had not increased in numbers. Virus injected into the common bile duct was never toxic, however much was given, and this is clearly because, as a result of the mechanics of the injection, only a limited number of hepatic cells are ever infected.

Livers taken at 18 hr. were also examined after routine haematoxylin and eosin staining. Necrotic periportal hepatic cells, which occasionally contained lymphocytes, were seen, and there was a periportal infiltration of mononuclear cells, most of which appeared to be in the lymphatic channels.

It was shown earlier that material which is toxic by intravenous injection is unable to infect hepatic cells when diluted more than 4-fold. The same material injected into the bile duct, however, still produced fluorescent hepatic cells when diluted 1 : 1000.

These experiments show that hepatic cells can readily be infected from the biliary channels but that the protective littoral cell barrier protects them from virus present in the blood, except when massive doses are injected.

EXPLANATION OF PLATES

FIG. 1.—Fluorescent antibody stained section of liver of mouse 5 min. after a massive intravenous injection of the MEL strain of influenza virus. Fluorescent littoral cells. ×165.

FIG. 2.—Fluorescent antibody stained section of liver of mouse dead 18 hr. after a massive intravenous injection of the PR8 strain of influenza virus. Scattered fluorescent hepatic cells. ×165.

FIG. 3.—Haematoxylin and eosin stained section of mouse liver after the injection of India ink into the bile duct. ×165.

FIG. 4.—Fluorescent antibody stained section of mouse liver 19 hr. after the injection into the bile duct of one-tenth the minimal toxic intravenous dose of the PR8 strain of influenza virus. Fluorescent hepatic cells round a portal tract. ×165.

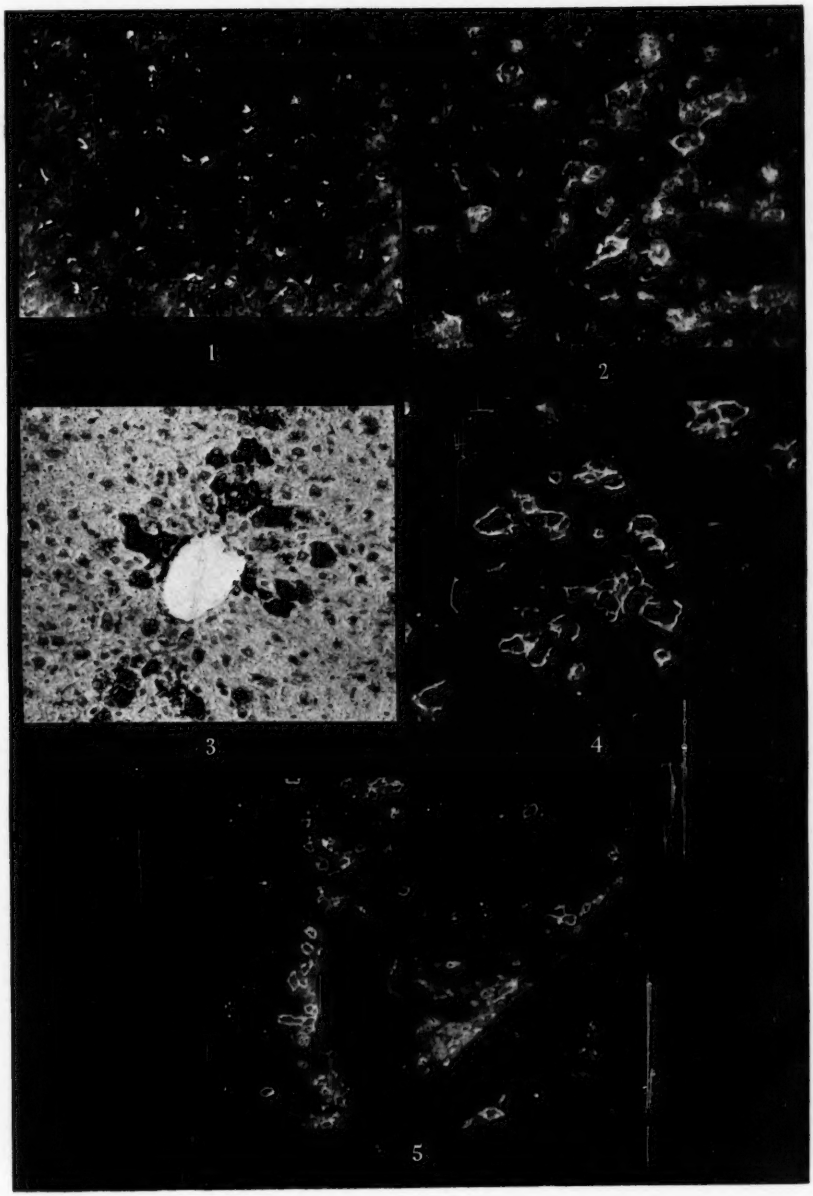
FIG. 5.—Same section showing fluorescent hepatic cells round a portal vein. ×110.

FIG. 6.—Fluorescent antibody stained section of suckling mouse carcass 46 hr. after the intraperitoneal injection of the MEL strain of influenza virus. Fluorescent foci in liver, and fluorescence of peritoneal surface of liver and neighbouring gut. ×66.

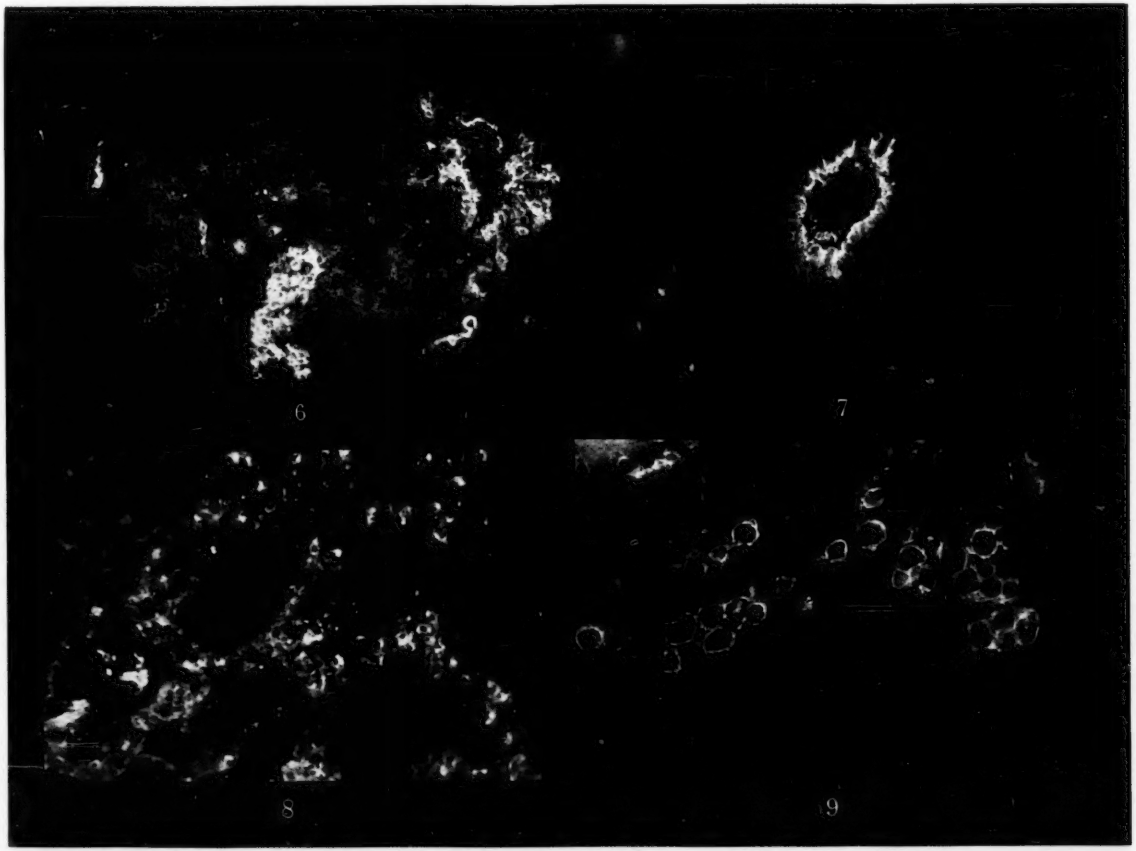
FIG. 7.—Fluorescent antibody stained section of suckling mouse lung 43 hr. after the intraperitoneal injection of the PR8 strain of influenza virus. A bronchiole, showing fluorescence of all epithelial cells, and a few fluorescent cells in the interstitial tissues. ×168.

FIG. 8.—Fluorescent antibody stained section of suckling mouse carcass 3 days after the intraperitoneal injection of the NWS strain of influenza virus. Scattered fluorescent cells in lung. ×133.

FIG. 9.—Fluorescent antibody stained section of leg muscle of suckling mouse 43 hr. after the injection into this muscle of the NWS strain of influenza virus. Fluorescent rims surround a number of muscle fibres. ×100.



Mims.



Mims.

The response of infant mice

Standard strains.—Large doses of virus were injected intraperitoneally to 1–4 day old mice. Deaths occurred within the first few days after injection, and whole carcasses and heads from sick mice were sectioned and stained. Foci of fluorescent cells were present in the liver, and the peritoneal surfaces of the diaphragm and viscera were brightly fluorescent (Fig. 6). The lungs, which were extensively consolidated, contained scattered fluorescent cells in the interstitial tissues and the entire epithelial lining of many bronchioles was brightly fluorescent (Fig. 7).

Neuro-adapted strains.—Large doses of the NWS strain were injected, and mice examined in the same way. Deaths occurred from the fourth day. There were extensive brightly fluorescent areas in the lungs (Fig. 8), and fainter fluorescent foci in the liver. No fluorescence was seen in the kidneys. Scattered small fluorescent foci were present in the brain parenchyma, and in some ventricular profiles nearly all ependymal cells were fluorescent. When virus was injected intramuscularly and the injected muscle examined two days later, a brightly fluorescent rim was seen round many muscle fibres (Fig. 9). It could not be decided whether this fluorescence was in the connective tissue sheath (endomysium) or in the cytoplasm of the muscle fibre itself.

DISCUSSION

It has been shown that standard strains of influenza virus grow in hepatic cells in the course of their so-called toxic intravenous action, and the rises in infectivity and haemagglutinin titres support the fluorescent antibody findings. Although most of the virus produced is non-infective the word " toxic " is clearly no more appropriate than it was in the case of intracerebral lethality. The fluorescent antibody used would be expected to react with haemagglutinin, but the haemagglutinin titres were lower than would have been expected from the amount of antigen seen in sections. Perhaps a haemagglutinin type antigen which does not haemagglutinate is produced.

The fact that the first fluorescent hepatic cells were seen as early as 7 hr. after injection indicates that virus reached these cells a short time after injection, presumably by " leaking " through the littoral cell lining of the sinusoids. From 8–18 hr., however, there was an increase in the number of fluorescent hepatic cells, and this could be because there were several cycles of growth in hepatic cells, or because the injected virus reached these cells at different times. Since foci of fluorescent cells did not develop, and since there was no evidence for more than one cycle of growth after bile duct injection, it is likely that virus from littoral cells " leaked " through to hepatic cells at different times.

There are about $10^{7 \cdot 5}$ littoral cells in the normal mouse liver, so that if these cells took up the greater part of a minimal toxic dose ($10^{8 \cdot 5}$ ID_{50}) each one would receive about 10 ID_{50}. There are more virus particles than there are ID_{50} and after a minimal toxic dose each cell might receive 50 or more particles. Smaller numbers than this are dealt with effectively, for antigen fades and does not reappear, but as the number of particles per cell is increased a level is reached at which virus grows in cells and at about this level most cells fail to prevent the infection of adjacent hepatic cells. This is the toxic dose level. When virus preparations containing a large proportion of non-infective particles are injected, the particles which reach hepatic cells are likely to be non-infective, and hence such preparations are non-toxic.

Hepatic cells readily support the growth of virus if it is introduced directly into the cells, via the bile duct. When a large amount of virus is injected intravenously, however, it is taken up by littoral cells and does not have an opportunity to infect hepatic cells. This means that the phagocytic littoral cells protect the susceptible hepatic cells from the microorganism, in the same way that they would phagocytose and digest effete erythrocytes in the sinusoids and thus prevent them from reaching hepatic cells. A similar protective action of the littoral cells has been encountered in the case of certain strains of vaccinia virus. Both the CL and CL-R strains are taken up by littoral cells after massive doses are injected intravenously, and most of the antigen in these cells has disappeared by 3 hr. By 10 hr. the CL-R strain has grown in many littoral cells, and this is followed by a limited infection of hepatic cells. There is no growth of the CL strain in littoral cells, which have thus in a sense protected hepatic cells from the virus, for this strain grows in hepatic cells when injected via the bile duct (Gemmell and Mims, unpublished observations). In mice infected with the Moscow strain of ectromelia (mousepox), on the other hand, the littoral cell protective action can be regarded as having failed, for a large proportion of the littoral cells support virus growth and then infect hepatic cells (Mims, 1959).

When a neuro-adapted strain was injected intramuscularly into infant mice there was growth in the injected muscle, and this confirms the findings of Wagner (1955). The fact that after the intraperitoneal injection of neuro-adapted strains there were extensive areas of ependymal fluorescence suggests that virus crosses the blood-CSF barrier at an early stage in the invasion of the brain.

The haemorrhagic enteritis which often accompanies the lethal action of intravenously injected influenza viruses is being investigated, and the findings will be published.

SUMMARY

The so-called toxic action of intravenously injected influenza virus has been shown to involve a single cycle of growth in hepatic cells. A rise in infectivity and haemagglutinin titres accompanies the production of antigen in cells.

Injected virus is taken up by littoral cells, but does not grow in these cells or infect hepatic cells unless massive doses are given, as a result of which each littoral cell receives a large number of infectious particles.

Hepatic cells are readily infected when virus is introduced into them directly, via the biliary tract.

Littoral cells may be thought of as protecting hepatic cells from circulating influenza virus.

The growth of extraneurally injected virus in infant mice is described.

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THE STIMULATION AND DEPRESSION OF RETICULO-ENDOTHELIAL PHAGOCYTIC FUNCTION BY SIMPLE LIPIDS

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THE cells of the reticulo-endothelial system (RES) in contact with the blood stream possess a singular degree of autonomy and the physiological factors concerned in the regulation of their activity remain mostly unknown. It is well recognised that RES phagocytic function may be altered by various colloids, certain infections and lipopolysaccharide from Gram negative bacilli (Biozzi, Halpern, Benacerraf and Stiffel, 1957). It must be emphasized that these substances are derived from widely different sources, are often chemically complex and are not physiological components of the organism. Accordingly it is of interest that certain simple molecules of known constitution can cause profound and opposing changes in phagocytic activity. This communication reports the effect of glyceryl and ethyl esters of fatty acids on phagocytic function as measured by the clearance of injected colloidal carbon from the blood stream.

MATERIAL AND METHODS

Mice

Adult males weighing 20–25 g. each were used.

Phagocytic index

The phagocytic function of the RES was investigated by measuring the rate of blood clearance of colloidal carbon (Guenther Wagner, Hanover) injected intravenously in a dose of 8 mg./100 g. (Biozzi, Benacerraf, Stiffel and Halpern, 1954). With this dose 95 per cent of the carbon is phagocytosed by the reticulo-endothelial cells of the liver and spleen. The kinetics of clearance follow an exponential function of the concentration to time according to the equation :

$$K = \frac{\log C_1 - \log C_2}{T_2 - T_1}$$

where C = blood concentration of carbon and T = time in min. The constant K which measures the total activity of the RES is called the phagocytic index.

The RES function has also been calculated by unit of weight of hepato-splenic tissue. This corrected phagocytic index α is obtained as follows :

$$\sqrt{K} \times \frac{W}{wls} = \alpha,$$

where W = body weight and wls = weight of liver and spleen.

Lipids

Emulsions of olive oil, glyceryl trioleate, glyceryl mono-oleate, ethyl stearate and ethyl oleate were prepared as follows : Tween 20 (final concentration 0·7 per cent) was mixed with

the lipid (final concentration 10 per cent) and the mixture added with vigorous stirring to 5 per cent aqueous glucose. It was mechanically mixed for 3 min. and injected intravenously in a dose of 0·25 ml./20 g. The great majority of the particles were less than 5 μ diameter only a few being larger than 10 μ. The mean particle size of the olive oil emulsion was 2·3 μ. In one experiment (Fig. 2) the emulsion had been prepared by Duncan Flockhart and Co., Edinburgh, as follows: Ethyl stearate 20 per cent, soya lecithin 1·2 per cent, pluronic F68 0·3 per cent, dextrose 3·2 per cent, water to 100 per cent. In this case, 0·016 ml./20 g. was given intravenously.

RESULTS

The effect of ethyl stearate and glyceryl trioleate on the clearance of colloidal carbon, measured 24 hr. after lipid administration, is shown in Fig. 1. The rate of

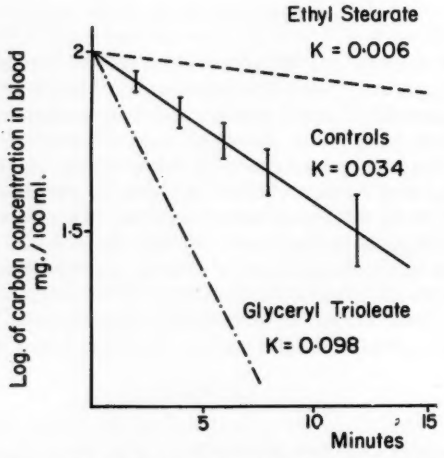

FIG. 1.—Showing opposing effects of ethyl stearate and glyceryl trioleate on rate of blood clearance of colloidal carbon measured 24 hr. after the lipid administration. The standard deviation is indicated for the controls.

carbon phagocytosis is markedly delayed after ethyl stearate and the phagocytic index K is about 6 times less than normal. On the other hand, treatment with glyceryl trioleate results in a threefold increase of phagocytosis.

Fig. 2 shows the duration of the depressant action of ethyl stearate on RES activity. A maximal effect is observed at 24 hr., as shown by the indices K and α. Thereafter phagocytic activity gradually recovers and reaches a normal value within 3 or 4 days. There are no significant changes in the weight of liver and spleen although perhaps a slight increase has occurred at 48 hr.

Similarly the duration of effect of an olive oil emulsion on RES function is seen in Fig. 3. This substance, like glyceryl trioleate, produces a marked increase in the RES activity as shown by the indices K and α. The effect is maximal at 24 hr. and remains at a high level for about 5 to 6 days after which it returns gradually to normal. There is no change in weight of liver and spleen.

The table summarizes the effect of these lipid emulsions on the RES. The phagocytic function has been measured 24 hr. after administration of lipid. Olive

oil and glyceryl trioleate produce a marked stimulation of phagocytosis. Glyceryl mono-oleate has no action whereas both ethyl oleate and ethyl stearate cause a severe depression of activity. In each case the corrected phagocytic index α parallels the index K since stimulating and depressing lipids alike produce no changes in size of liver and spleen.

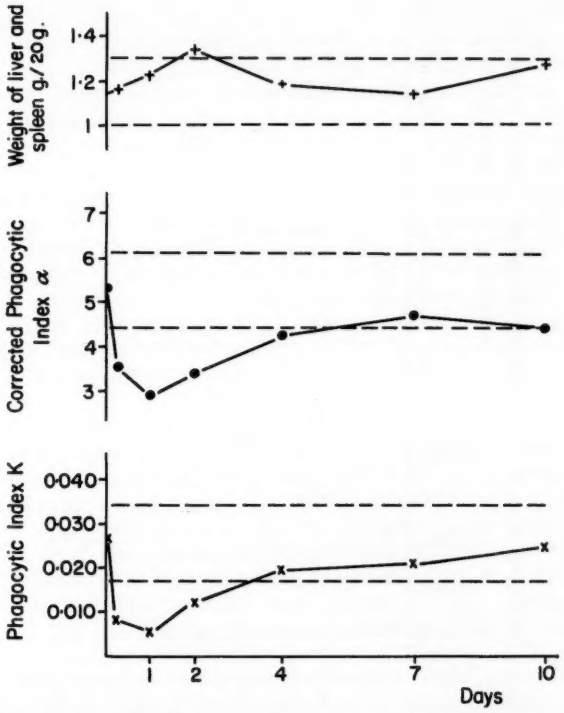

FIG. 2.—Duration of depression of the phagocytic activity of the RES following the injection of ethyl stearate given at zero time. Each point is the mean value obtained in 10 mice. Broken lines indicate the limits of the normal standard deviations.

DISCUSSION

It seems likely that these substances act mainly on the afferent or receptor side of the reticulo-endothelial system where they modify the function of the sessile phagocytic cells. Direct histological examination of the liver and spleen, after the injection of a fatty emulsion, has shown fat droplets in the Kupffer cells (Jaffe and Berman, 1928). Our own experiments with isotope labelled lipid show that 50 per cent of the radioactivity is found in the liver and spleen. These findings suggest that the functional changes described here are due to the intracellular accumulation of fat and the chemical nature of the fat determines the effect produced.

The stimulating activity is very marked and in some instances the index of phagocytic function was increased tenfold. This effect could be produced in about 80 per cent of animals and the reason for failure of stimulation in the remainder is unknown. Since this action occurs early and is not accompanied by an increase in the weight of the liver and spleen, it seems unlikely that cell multiplication by

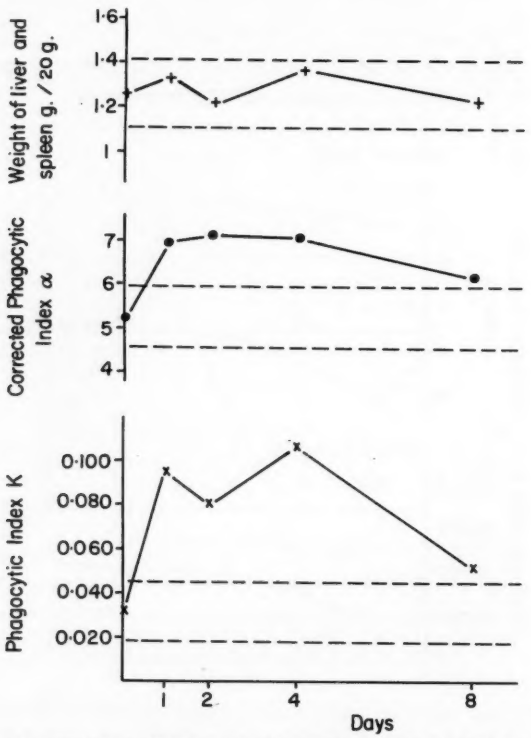

FIG. 3.—Duration of stimulatory effect on the phagocytic activity of the RES following the injection of olive oil given at zero time. Each point represents the mean value established in 10 mice.

Broken lines indicate the limits of the normal standard deviations.

TABLE.—*Effect of Different Lipid Emulsions on the Phagocytic Indices K and α, 24 hr. after the Lipid Administration*

Lipid	Number of animals	K	α	Weight of liver and spleen (g./20 g.)
Olive oil	5	0·095	7·0	1·310
Glyceryl trioleate	10	0·098	7·4	1·230
Glyceryl mono-oleate	6	0·036	5·3	1·250
Ethyl stearate	8	0·006	3·0	1·220
Ethyl oleate	12	0·018	4·0	1·315
Controls	21	0·034±0·013	5·2±0·8	1·230±0·100

itself is the cause of hyperactivity. On the contrary, there occurs cellular hyperfunction which results in an increase of surface trapping and subsequent transference of particulate matter to the cell interior.

No explanation can be offered for the wide difference in physiological activity between the mono-oleate and trioleate of glycerol although it is of interest that the addition of two further oleic residues can so markedly affect the biological activity of the molecule.

The depressant action of the ethyl esters of oleic acid and stearic acid is profound and the question arises whether this effect is due to mechanical saturation of the target cells resulting in inability to deal with the test dose of carbon. If this were so, one would expect all the lipid emulsions described here to have a similar effect because they contain roughly equal numbers of particles, However this is clearly not the case and furthermore very small amounts of emulsified lipid (as little as 2 mg./20 g.) can produce a marked effect. In addition blockade by saturation with inert colloids is usually of short duration and is followed by a phase of overactivity (Biozzi *et al.*, 1957). By contrast the oleate and stearate compounds discussed here possess a rather more prolonged action which may be sustained by repeated injections. For these reasons it is unlikely that mechanical blockage plays a significant part ; it remains that these substances have a direct cytotoxic effect, a view supported by Shivas and Fraser (1959) who noted a destructive effect on lymph nodes following intraperitoneal injection. However lymph node cells are not quite the same as Kupffer cells which are notoriously resistant to injury.

The contrasting effects produced on RES function by simple lipids makes them particularly useful tools for exploring the effect of phagocytosis on immunity to infection, antibody production and related problems. It is remarkable that this group of compounds possess any activity whatsoever because they are not dissimilar from the fatty aggregates which appear in the blood after a meal. Chylomicron fat is removed from the circulation (French and Morris, 1957) according to an exponential function identical with that observed in other colloids phagocytosed by the RES. Nevertheless the latter authors are not quite prepared to accept a direct action of the RES on fat clearance, although there is some evidence for such a direct action (Byers, Mist-St. George and Friedman, 1957). Because we have found opposing effects with esters of certain fatty acids and because these acids are normal constituents of the tissues, it is tempting to believe that circulating blood lipid exerts a measure of control on RES activity.

Perhaps of chief interest is the simplicity of the molecules which produce these contrasting effects. This appears to create a new approach to the perennial problem of chemical structure and biological activity. At the moment we can conclude only that the ethyl esters of certain fatty acids depress whereas glyceryl esters stimulate phagocytic function. It is hoped that further examination of suitably tailored molecules will contribute to the understanding of this basic physiological process.

SUMMARY

The total phagocytic function of the RES in adult mice has been measured quantitatively by the estimation of clearance of colloidal carbon from the blood stream. The effect of certain emulsified lipids on the rate of phagocytosis has been investigated. Substances such as olive oil and glyceryl trioleate cause an

intense stimulation of phagocytic function; glyceryl mono-oleate has no effect whereas ethyl oleate and stearate markedly depress this function.

We wish to thank Dr. A. A. Shivas, Dept. of Pathology University of Edinburgh, and Dr. W. T. Simpson of Duncan Flockhart Research Laboratories, Edinburgh for valuable assistance. One of us (A. E. S.) wishes to acknowledge the aid of a University Travelling Scholarship and Ciba Anglo-French Bursary Award.

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AMYLOIDOSIS IN MICE FOLLOWING INJECTIONS WITH FREUND'S ADJUVANT, ITS COMPONENTS SEPARATELY AND HOMOLOGOUS LIVER-ADJUVANT MIXTURE

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In a previous investigation on the effect of Freund's adjuvant and its components on the organs of various experimental animals it was found that the injection of Freund's adjuvant into mice resulted in the deposition of amyloid in the spleen, liver and kidneys (Laufer, Tal and Behar, 1958).

The present study on mice was undertaken in order to determine which of the components of the adjuvant was responsible for the amyloid deposition; to ascertain the minimum number of injections of one of the components or of the complete adjuvant needed for the above mentioned reaction, and the time required for its development; and to study the histopathological changes resulting from the addition of liver emulsion as an isoantigen, to the adjuvant (Behar and Tal, 1959), in view of the suggestion that the production of amyloid in mice is an immunological response (Grayzel, Grayzel, Miller, Cohen and Akst, 1957).

MATERIALS AND METHODS

Mice, inbred, W-Swiss, H-line, 6 weeks old, of both sexes, were used, weighing 20–25 g. each. The Freund's adjuvant and fractions were prepared as before (Freund and McDermett, 1942; Olitsky and Tal, 1952), and were injected subcutaneously in 0·3 ml. doses. One hundred and forty-one animals were divided into 8 groups. Group I (17 animals) received one single injection of whole adjuvant. Group II (32 animals) received 6 weekly injections of whole adjuvant, Group III (9 animals) received one single injection of the *Mycobacterium tuberculosis* fraction (Tbc fraction). Group IV (20 animals) received 6 weekly injections of the Tbc fraction. Group V (10 animals) received one single injection of the oil fraction of the adjuvant. Group VI (15 animals) received 6 weekly injections of the oil fraction. Group VII (19 animals) received one single injection of whole mouse liver plus adjuvant. Group VIII (19 animals) received 6 weekly injections of whole mouse liver plus adjuvant.

All animals in the above groups were killed 8 weeks after the first (or only) injection.

Forty-eight additional mice were divided into 4 groups of 12 in each group. These groups received 6 weekly injections of whole adjuvant, Tbc fraction, oil fraction, and whole adjuvant plus liver, respectively, but 2 animals of each group were killed each week.

Ten additional mice were kept under the same conditions as the injected mice and were used as controls. All the animals were killed by ether inhalation.

Blocks for the histological examination of tissue were taken from the spleen, liver, kidney, and brain. The organs were fixed in 10 per cent neutral formalin and were then embedded in paraffin, stained with haematoxylin and eosin, Congo red, PAS, and Gentian violet.

RESULTS

In Table I the number of animals which developed amyloidosis in the various groups killed at 8 weeks is shown.

TABLE I.—*Development of Amyloid in Animals Killed 8 Weeks After Injection*

Group	Material	Number of injections	Number of animals	Number of amyloid	Results after 8 weeks: Time of appearance (weeks)	Results after 8 weeks: Number of injections to appearance
I	Whole adjuvant	1	17	2	8	1
II	,, ,,	6	32	32	8	6
III	Tbc	1	9	0	—	1
IV	,,	6	20	7	8	6
V	Oil	1	10	0	—	1
VI	,,	6	15	2	8	6
VII	Liver + adjuvant	1	19	1	8	1
VIII	,, ,, ,,	6	19	19	8	6

Even a single injection of whole adjuvant resulted in the production of amyloid in occasional animals after 8 weeks. However, after repeated injections of whole adjuvant all animals showed amyloid deposits.

A single injection of the Tbc fraction of the adjuvant did not result in amyloid production. Repeated injections of the Tbc fraction gave positive results in 7 out of 20 animals.

The oil fraction gave negative results after one injection, but amyloid developed in 2 of 15 animals who received repeated injections of this fraction.

A single injection of whole mouse liver plus adjuvant resulted in amyloid production in one of 19 animals, whereas all animals showed amyloid after repeated injections of liver plus adjuvant. No histological evidence of liver necrosis was, however, found.

In Table II the time of appearance of amyloid in the animals killed at weekly intervals after repeated injections is shown. Only 2 mice were killed at each week in each group, and in view of a possible varying sensitivity of individual mice the results must be interpreted with caution.

TABLE II.—*Appearance of Amyloid in Animals Killed at Weekly Intervals After Injection*

Group	Material	Week	Number with amyloid
II	Whole adjuvant	1	0/2
		2	1/2
		3	0/2
		4	2/2
		5	2/2
		6	2/2
IV	Tbc fraction	1–4	0/2
		5	1/2
		6	2/2
VI	Oil	1–6	0/2
VIII	Liver + whole adjuvant	1	0/2
		2	1/2
		3	1/2
		4	1/2
		5	2/2
		6	2/2

In animals receiving whole adjuvant or whole adjuvant plus liver, amyloid was already encountered in mice killed 2 weeks after the first injection. In mice receiving only the Tbc fraction, amyloid was present in 1 of 2, killed 5 weeks after the first injection, and in both mice killed after 6 weeks, but in none of the mice killed before the 5th injection. Of 12 mice who received the oil fraction in this experiment none developed amyloid.

Histological examinations

The pertinent findings were the presence of amyloid in the liver, spleen and kidney. The intensity of amyloidosis varied in the different animals. The spleen appeared to be the first organ to react. It was affected in all positive cases, and in some animals it was the only organ to show amyloid deposits. The liver was affected less frequently than the spleen, and the kidney the least frequently of the 3 organs.

In some animals, the structure of the spleen was completely destroyed and replaced by large deposits of amyloid (Fig. 1), whereas in others the deposit was mainly in the follicles. The cellular pattern of the pulp was mostly composed of lymphoid cells, giant cells, histiocytes and rare plasma cells. There was never an abundance of plasma cells even in animals where very scanty deposits of amyloid were found. The amyloid stained positively with Congo red and Gentian violet.

In the liver the amyloid was usually present along the sinusoidal linings (Fig. 2). Larger deposits of amyloid produced atrophic changes in the liver trabeculae and in some areas the trabeculae were destroyed and replaced by the deposits of amyloid. Amyloid was also deposited in the vessel walls. In many sections of the liver in the various groups, the presence of a cellular infiltration consisting mainly of lymphoid cells, histiocytes and a few leucocytes was noted. This reaction of the reticulo-endothelial elements was present in the portal spaces, around central veins and extending into the parenchyma of the liver (Fig. 3). No correlation could be made between the presence or intensity of these cellular proliferations and the deposition of amyloid.

The amyloid in the kidneys, if present, was deposited in the glomeruli. A few animals showed, in addition, a mild interstitial infiltration with lymphoid cells. Amyloid was never found in the kidney in the absence of splenic or hepatic amyloidosis.

DISCUSSION

The basic mechanism for the production of amyloidosis remains unknown. Certain investigators have suggested that the amyloid is the result of an altered allergic or hypersensitivity state (Grayzel *et al.*, 1957). In our experiments in which fractions of Freund's adjuvant were injected, amyloid deposition was mainly associated with repeated injections of the Tbc fraction. The need for repeated injections of the Tbc fraction rather than a single one, supports the above mentioned hypersensitivity hypothesis. Occasionally a single injection of whole adjuvant could also produce amyloid deposition. This could also be regarded as a hypersensitivity state, caused by a continuous sensitization from the local adjuvant deposit (Freund, 1957). In the 1st experiment the number of mice which developed amyloidosis after repeated injections of the Tbc fraction (7 out of 20) seemed to be higher than in animals receiving the oil fraction only (2 out of

15). In the 2nd experiment 3 of 4 animals killed at least 5 weeks after repeated Tbc injections showed amyloid, whereas none of those who received the oil fraction developed amyloid. These results suggest that the active sensitizing agent of Freund's adjuvant is the Tbc fraction, which agrees with the findings of Rothbard and Watson (1954). We have no ready explanation for the development of amyloidosis in 2 of the mice which received oil only. In other experiments we have given a further number of mice repeated injections of the oil fraction and in none of these did amyloid appear.

Amyloid deposition is less frequent in the animals receiving repeated injections of the Tbc fraction only than in the group receiving the whole adjuvant. This suggests that the oil fraction of the adjuvant also plays a role in the formation of amyloid, possibly by prolonging the action of the Tbc fraction, or by influencing its distribution, or both.

In spite of the assumption that the amyloid formation is an immunological response, no increased number of plasma cells was found at the stage when amyloid had already been deposited. This finding does not, however, negate the importance of the plasma cells in the earliest stages of the formation of amyloid (Ehrich, 1952).

In hamsters and guinea pigs injections of a mixture of liver emulsion and Freund's adjuvant result in liver necrosis but no amyloid deposition (Behar and Tal, 1959). Mice, on the contrary, develop amyloidosis and show reticulo-endothelial proliferation but no evidence of liver necrosis. This species difference may be due to the fact that in mice, which react to immunological stimulus by amyloid production, the union of antigen and antibody does not occur within the liver cells so that liver cell necrosis does not result (Lucke and Markley, 1928).

SUMMARY

Single and repeated injections of Freund's adjuvant in mice result in amyloid deposits in spleen, liver and kidney. Repeated injections of the Tbc fraction of the adjuvant caused the deposition of amyloid in the same organs. but in a lower percentage of animals. Repeated injections of the oil fraction produced amyloid deposits in 2 out of 15 animals, while a single injection of either fraction did not cause amyloid deposits.

Repeated injections of liver homogenate plus Freund's adjuvant gave the same results as repeated injections of whole adjuvant, but no liver damage was detected. A single injection of liver homogenate plus adjuvant gave positive results in 1 out of 19 animals.

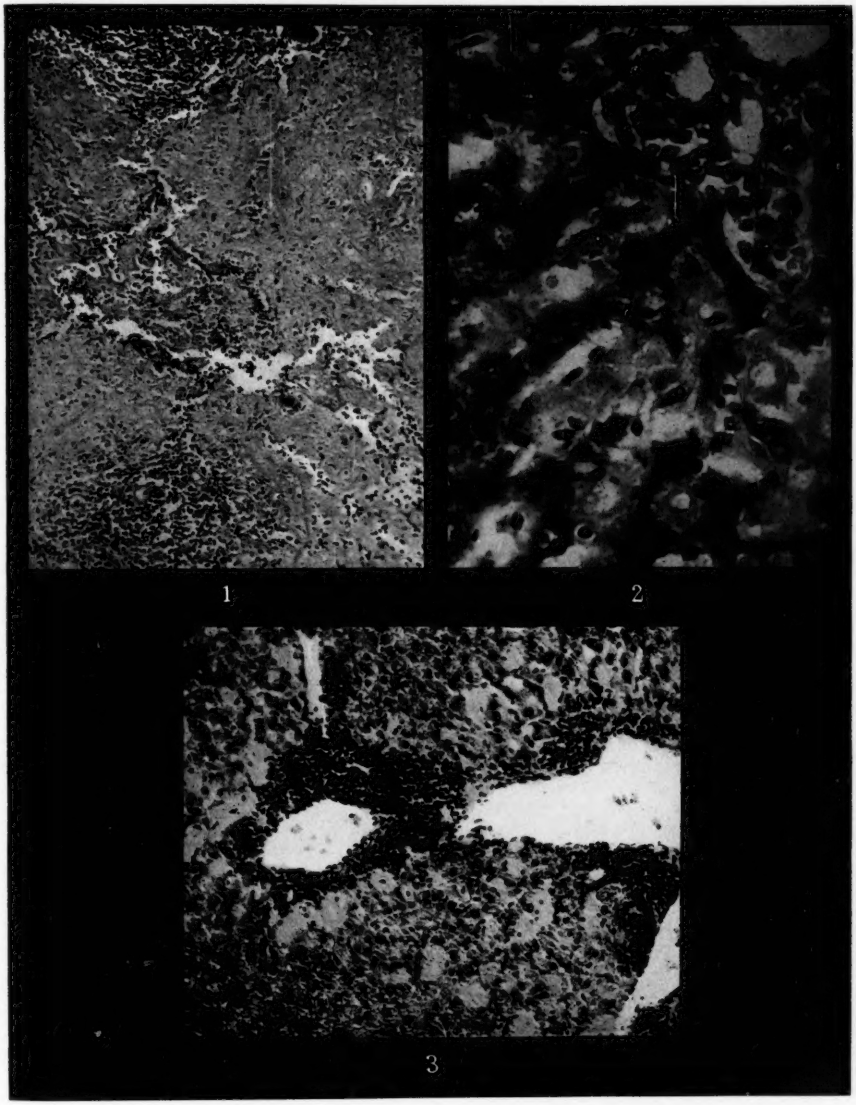
Amyloid could usually be found after the 2nd weekly injection but its appearance was delayed until after the 5th week in animals given the Tbc fraction alone.

EXPLANATION OF PLATE

FIG. 1.—Large amyloid deposits in the spleen where only remnants of follicles are present. H.E. ×94.

FIG. 2.—Amyloid deposit along the sinusoidal lining; with atrophy of liver trabeculae. H.E. ×376.

FIG. 3.—Reticulo-endothelial cell reaction in the liver. H.E. ×94.



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These findings are discussed in relation to the pathogenesis of amyloidosis and the specificity of the animal species' reaction.

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SOME PROPERTIES OF A STRAIN OF SV17 VIRUS ISOLATED FROM AN EPIDEMIC OF CONJUNCTIVITIS AND RHINORRHOEA IN MONKEYS (*ERYTHROCEBUS PATAS*)

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A virus serologically identical with SV17 virus (Hull, Minner and Mascoli, 1958) has been isolated (Goffe, unpublished) from the nasal and conjunctival secretions of patas monkeys which were living in captivity under conditions of partial isolation and were affected by a disease resembling to some extent pharyngoconjunctival fever of man (Bell, Rowe, Engler, Parrott and Huebner, 1955). The circumstances under which the virus was isolated indicated that the virus caused the disease. The present paper summarizes experiments on the behaviour of this virus in laboratory hosts and certain of the basic properties of the virus particle.

MATERIALS AND METHODS

Tissue cultures.—Rhesus and cynomolgus monkey kidney cultures were prepared in test tubes from trypsinized cell suspensions (Melnick, 1955). Cultures were maintained at 36° in a roller drum in 1·5 ml. of either medium 199 of Parker or a mixture of 2 per cent calf serum and 0·25 per cent lactalbumin hydrolysate in Hanks' saline with antibiotics.

Virus titrations.—Serial 10-fold dilutions of virus were inoculated into groups of 2–4 roller tube cultures which were observed for cytopathic effects for 10–14 days. Interpolated 50 per cent infectivity endpoints were calculated.

Haemagglutination tests.—Serial 2-fold dilutions of virus were made in 0·25 ml. volumes in cups in " perspex " plates using saline buffered at pH 7·1 with 0·01 M phosphate buffer. Red cells were collected in acid citrate dextrose solution, washed and stored as 10 per cent suspensions in dextrose, gelatin veronal solution (Clarke and Casals, 1958). The suspensions were diluted 1/10 in buffered saline and equal volumes were added to the virus dilutions. The cells were allowed to settle at room temperature and partial agglutination was taken as the endpoint. Virus pools consisted of the fluid and detached cells of infected cultures stored at −20° or −60°.

Almost all experiments on the infective property of the virus were performed with the virus from cultures which had been inoculated with eye or nasal swabs from monkeys. Material from later passages was used in many experiments on haemagglutination.

pH measurements were made with a glass electrode instrument.

Fluorescent antibody staining.—Cultures were fixed in absolute alcohol and treated firstly with pooled convalescent monkey serum absorbed with normal tissue and secondly with guinea pig complement. Complement was detected with fluorescein conjugated anti-guinea pig globulin from immunized rabbits (Goldwasser and Shepard, 1958).

Cytological methods.—Cultures fixed in absolute alcohol were stained with acridine orange (Armstrong, 1956) and Mayer's haemalum. The changes in distribution of nucleic acid seen in acridine orange stained cells were confirmed by staining by Feulgen's method and with methyl green pyronin (Trevan and Sharrock, 1951).

RESULTS

The host range of the virus

In tissue culture.—The presence of virus was recognized by a striking rounding up of cells in tissue cultures of rhesus and cynomolgus monkey kidney. The cells became yellowish and intensely granular and tended to form clumps or clusters. This appearance was seen first around the edge of the cell sheet when large doses of virus were given but began focally when small doses were given. After a day or two all the cells were affected and floated off in the medium. A similar degeneration occurred when large doses (about 10^5 $TCD_{\cdot 50}$) were inoculated into primary cultures of human amnion and human embryo kidney cells and also of calf kidney cells. Degeneration also occurred after inoculating HeLa cells. The changes in calf kidney were not complete but were transmitted serially through 5 passages with a gradually increasing latent period. There may therefore have been some virus multiplication but the virus was only poorly adapted to these cells. Similar doses of virus caused partial degeneration of explant cultures of chick embryo lung epithelium but no changes in chick embryo heart fibroblasts.

In experimental animals.—Guinea-pigs, weanling and suckling mice, and embryonated eggs of various ages were inoculated by various routes with about 10^6 TCD_{50} of virus. No disease or gross pathological changes were observed and there was no evidence of virus multiplying in the tissues.

Stability of virus infectivity

The virus seemed in general to be very stable. In particular it was held overnight at 4° with 20 per cent anaesthetic ether and the infectivity titre was the same as that of a control sample—$10^{4\cdot2}$/ml. In another experiment a virus pool was diluted 1/10 in 8 different 0·1 M buffers giving final pH values ranging from 2·6 for a citric acid-citrate buffer to about 10·5 for a NaOH-glycine buffer. After 1 hr. at 37° each buffer mixture was diluted 10-fold further into 2 per cent horse serum in 0·1 M phosphate buffer pH 7·1. The infectivity titre of all these mixtures was identical—$10^{3\cdot5}$/ml. It was concluded that under the conditions tested, virus infectivity was unaffected by ether treatment or by pH between 2·6 and 10·5.

Ultrafiltration and electron microscopy

Virus pools were diluted 1 : 10 in 5 per cent inactivated horse serum in Hanks' saline and filtered through Gradocol membranes. The filtrates and unfiltered material were titrated in tissue cultures. In 3 separate experiments virus passed 2 filters of average pore diameter (APD) 100 mμ. No virus was detected after passage through one filter 93 mμ APD and 2 filters APD 91 mμ—the unfiltered fluid had a titre of 10^6/ml. According to Black (1958) the best estimate from these data of the diameter of the infectious particle is 62 mμ.

The sedimentation of the virus particle in a gravitational field was studied using the S40 head of the Spinco model L centrifuge and a modification of the sucrose density gradient method used by Melnick, Rhian, Warren and Breese (1951). This work will be presented in detail elsewhere but all samples contained a major haemagglutinating and infectious component with a sedimentation constant in distilled water of 350 Svedberg units. There were also more rapidly sedimenting components which probably represented aggregates.

Tissue culture fluids were mixed with neutral formalin, latex spheres and neutralized phosphotungstic acid and sprayed on to grids (Brenner and Horne 1959). Virus-like particles were detected in clusters and singly. They were readily detected in purified and concentrated virus prepared by adsorption on to zinc hydroxide (Newton and Bevis, 1959), sedimentation to form a pellet in the ultracentrifuge (60,000 *g* for 30 min.) and subsequent resuspension in distilled water and freezing and thawing.

Fig. 1 and 2 show the main features observed. The particles seemed to be 6-sided in outline and measured 65 mμ in diameter and thus were apparently identical in shape and size with the adenoviruses. Assuming that the infectious component of the haemagglutinin with sedimentation constant of 350 *S* had a particle diameter of 65 mμ then from Stokes' equation the density was calculated to be 1·38.

Multiplication of virus in rhesus monkey kidney cells

Test tubes containing coverslips were inoculated with $10^{6.3}$ TCD_{50} of virus. After one hour at 37° the cultures were washed 5 times with Hanks' saline and the medium was then replaced. At intervals thereafter medium was removed from the cultures, which were either fixed in alcohol at −60° or stored without alcohol at −20°. Later the unfixed cells were ground up, coverslip and all, with powdered glass, and the suspension was titrated by haemagglutination and in tissue cultures, as also was the medium. The fixed cultures were stained with haematoxylin and eosin and for nucleic acids by the acridine orange method, or for virus antigen by the fluorescent antibody method. The result of such an experiment is summarized in Fig. 3. From this it can be seen that a small and unchanging amount of infectious virus could be detected in the cells up to 10 hr. after inoculation. There was then a rapid rise and haemagglutinin could be detected from the 14th hour when the infectivity titre reached about 10^4. Virus appeared in the medium at a fairly late stage, probably when cells became detached from the sheet. Occasional cells showed specific virus antigen staining about 14 hr. after infection ; the number of affected cells and the intensity of staining both increased up to 18–20 hr. At this time early cytopathic changes were first detected in unstained preparations in the form of scattered rounded cells, especially at the edge of the culture.

The fluorescent antibody staining was seen first as a uniform staining of the nucleus. Later the staining appeared irregularly mottled or else diffuse with a greater intensity towards the edges of the nucleus (Fig. 4). Finally in rounded cells the cytoplasm was stained and there seemed to be unstained areas inside the nucleus (Fig. 5).

The first morphological changes were seen in stained preparations fixed at about 14 hr., when antigen was first detected in the cells. All the early changes were seen in the nuclei : the distribution of chromatin (as seen in preparation stained with haematoxylin and eosin) or of nuclear DNA (as seen after acridine orange staining) was altered, becoming uniform or coarsely granular, and generally more dense. In advanced stages the nucleoli were indistinguishable (Fig. 6).

In cultures fixed with alcohol and stained with haematoxylin and eosin small eosinophilic bodies could be seen distributed throughout the nucleus (Fig. 7). Sometimes these were surrounded by a " halo " of almost unstained nuclear substance. Small vacuoles were seen especially near the nuclear membrane and the nucleolus. These were larger in preparations fixed at a later stage of infection.

They did not appear to contain nucleic acid. In 18 hr. cultures a few cells were seen in which the chromatin formed a mass in or near the centre of the nucleus and this mass was surrounded by an unstained space (Figs. 8 and 9). The central mass usually stained intensely for DNA but occasionally showed a tint suggesting the presence of RNA. Protein " crystals " were not seen.

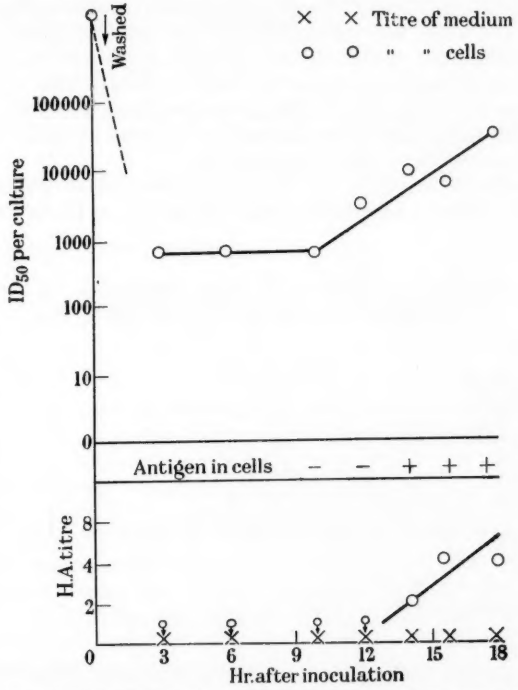

FIG. 3.—The early stages of the growth of virus in tissue cultures of rhesus monkey kidney cells. Virus antigen was detected in cells by the fluorescent antibody method.

In summary, after a latent period of 12–14 hours virus antigen appeared in the cell nucleus and infectious units could be extracted from the cells. There was a marked change in the distribution of nucleic acid in the nucleus and apparently an increased content of DNA. Later gross degenerative changes occurred in the nucleus ; these on the whole resembled those seen in cells infected with adenovirus types 3, 4 and 7 (Barski and Cornfeit, 1958 ; Boyer, Denny and Ginsberg 1959) and the cells then rounded up and were readily detached from the glass.

Haemagglutination

Fluids from virus infected cultures were mixed with equal volumes of red cells. Agglutination was observed with human, ox, rhesus monkey and sheep cells, but not with horse, fowl or goose cells. Low titres under 10 were obtained

with rhesus monkey cells but titres up to 256 were obtained with human cells; however there was usually a curious zone phenomenon in that dilutions of up to 1/8 of tissue culture fluid did not agglutinate. It was observed that this could be removed by treating the culture fluid with Arcton 63 (Pereira, Allison and Balfour 1959) or with trypsin. The effect was not seen with virus adsorbed on to calcium phosphate and eluted with phosphate buffer (Taverne, Marshall and Fulton 1958). The medium constituents had no haemagglutination inhibiting activity and it was concluded that the zone was probably due to a protein released from infected cells. Haemagglutination inhibition tests using 4–8 units of virus were carried out by methods like those used with influenza viruses. Serum from a rabbit immunized against prototype SV17 virus inhibited haemagglutination to a titre of 1/640, and well marked increases in titre were detected in the sera of many monkeys affected in the epidemic outbreak (Goffe, unpublished).

We next studied the effect on haemagglutination of exposure of virus to various chemical reagents and some results are shown in the lower half of Table I.

TABLE I.—*Effect of Various Treatments on the Haemagglutinating Activity of the Virus*

Treatment	Titre of sample	
	Untreated	Treated
Heating at 56° for 20 min.	128	<4
,, ,, 37° ,, 21 hr.	384	128
Exposure at room temperature for 60 min. to pH 2·2	128	64
pH 6·8	128	64
pH 10·0	256	256
Sodium metaperiodate (M/42·8) at room temperature for 1 hr.	64	48
Sodium metabisulphite (M/19) at room temperature for 1 hr.	192	192
Adsorption to calcium phosphate and elution in phosphate buffers pH 7·1		
0·1 M	256	16
0·5 M	256	64
Trypsin 0·01 per cent at 37° for 2 hr.	128	128
Chymotrypsin 0·01 per cent at 37° for 2 hr.	128	256

It can be seen that the haemagglutinin resists oxidation, reduction and treatment with proteolytic enzymes. In spite of the remarkable resistance shown to some agents it was found that haemagglutinating activity was quite heat labile. Culture fluid heated at 56° for 10 min. showed no haemagglutinin—a 64-fold drop in activity. Tissue culture fluid was mixed with an equal volume of 1 M buffers to give final pH values ranging from 2·2 to 10. After 1 hr. at room temperature the fluids were dialysed against buffered saline for 3 hr.: the haemagglutinin titres were unchanged.

The stability of the virus receptor on the red cells was also studied; in particular we tested the effect of treating red cells with cholera filtrate, trypsin and formalin. It can be seen from Table II that whereas myxovirus receptors were destroyed by cholera filtrate and not by formalin, ECHO 11 virus receptors were destroyed by formalin and not by cholera filtrate while adenovirus 9 and patas virus receptors were destroyed by both reagents. A further and more detailed report on these experiments will be presented elsewhere.

Finally it was found that the ratio between haemagglutinin titre and infectivity titre in tissue cultures was about $10^{4 \cdot 5}$.

TABLE II.—*Effect of Various Treatments on the Agglutinability of Human Red Cells by Various Viruses*

Treatment	Agglutination by			
	Influenza	ECHO virus type 11	Adenovirus type 9	Patas monkey virus SV 17
Incubation at 37° for 2½ hr. with cholera filtrate diluted				
1 in 640	−*	+	−	+
1 in 160	−	+	−	−
1 in 40	−	+	−	−
Incubation at 37° for 16 hr. with trypsin 0·01 per cent	32†	32	256	32
Untreated control	64	64	512	64
Incubation at 37° for 16 hr. with chymotrypsin 0·01 per cent	32	<2	256	64
Untreated control	64	64	512	64
Buffered formalin 1·0 per cent for 16 hr.	400	<2	300	<2
Untreated control	600	160	6400	256

* Presence or absence of agglutination by 8 units of virus, measured with untreated cells.
† HA titre obtained using treated cells.

DISCUSSION

During this study we were impressed by the ways in which the virus resembled the adenoviruses affecting man. The type of degeneration in cultures was similar to that produced by adenoviruses and, like them, the virus failed to grow in the usual laboratory animals, other than the monkeys. It seemed to multiply within the cell nucleus and not to be immediately released from the cells. The size, shape and density of the virus corresponded closely with those of the adenoviruses for which such data are available (see Pereira 1959). As far as it was tested, the red cell receptor for the virus behaved like that for adenovirus type 9, and the stability of the virus haemagglutinin was almost identical with that of adenovirus type 9 (Buckland and Tyrrell unpublished). In addition to these laboratory properties the virus caused a disease of monkeys like a common form of adenovirus infection of man. The resemblance to human adenoviruses was made complete when, towards the end of this work it was found that the virus produces a complement fixing antigen reacting with convalescent sera from cases of adenovirus infection in man (Goffe, unpublished).

SUMMARY

The virus under study multiplied in cultures of monkey kidney cells. After a latent period of 12–14 hr. virus antigen was detected in the nucleus, and infectious virus and haemagglutinin were detected in disintegrated cells. The degenerative changes which followed resembled those produced by adenoviruses.

The virus had an apparent diameter by filtration of 62 mμ and by electron microscopy of 65 mμ. Infectivity was stable to treatment over a wide range of pH and with ether.

The haemagglutinin seemed also to be very stable to treatment with a variety of chemical agents. It was neutralized by specific virus antiserum and sedimented in the ultra-centrifuge at about the same speed as infectious activity.

We wish to thank Major L. Kent for conjugating our immune globulin with fluorescein. We wish to thank Miss P. Pearce, Mrs. E. Beale and Mr. W. J. Randles for technical assistance.

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EXPLANATION OF PLATES

FIG. 1.—A concentrated suspension as seen by electron microscopy after a "negative stain" treatment with phosphotungstic acid. Virus particles of uniform size can be clearly identified despite the presence of debris. ×100,000.

FIG. 2.—The 6-sided profile of a virus particle shown by direct staining with phosphotungstic acid. ×200,000.

FIG. 4.—Changes in rhesus monkey kidney cells. Preparation fixed in absolute alcohol 18 hr. after virus inoculation. Stained by the fluorescein conjugated antibody method. The nucleus is diffusely stained. Staining is more intense near the nuclear membrane. ×1160.

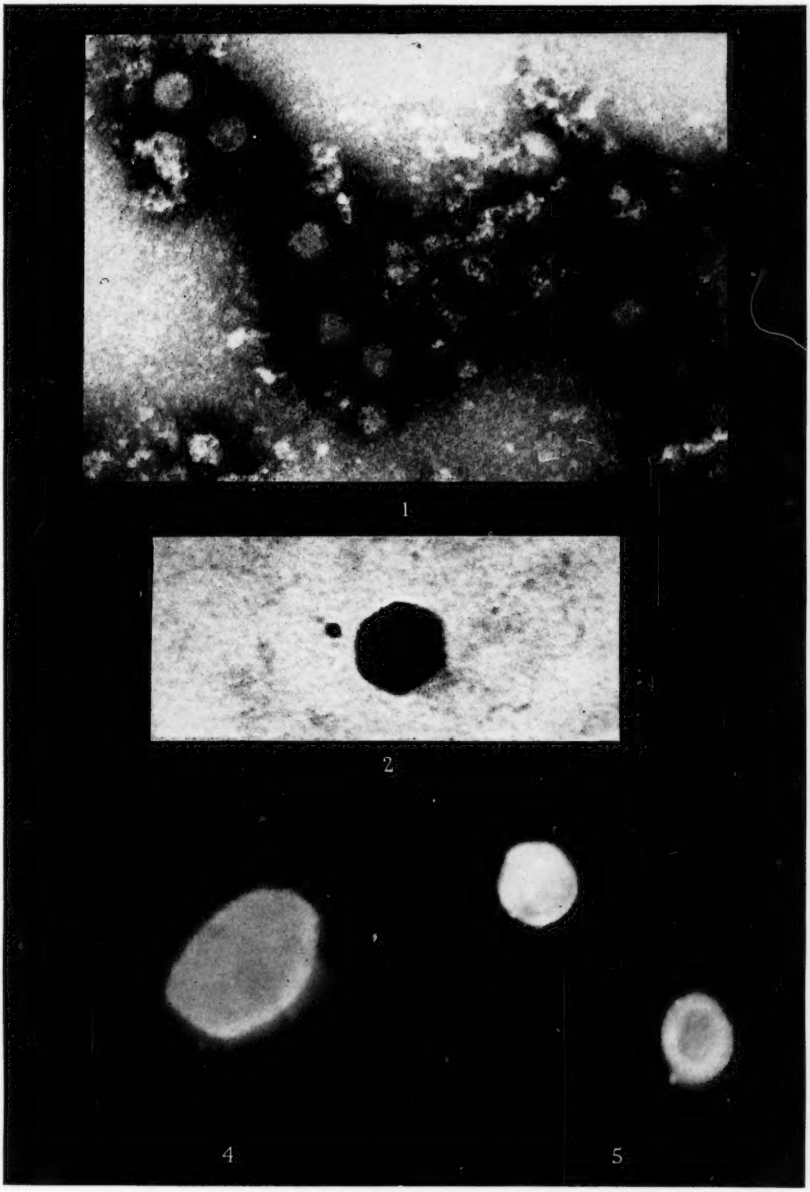
FIG. 5.—As Fig. 4. Cynomolgus kidney cells 19 hr. after infection. More advanced changes and fluorescence in the cytoplasm. ×517.

Fig. 6.—Cells 24 hr. after infection. Moderately advanced changes in the nuclei. Loss of normal chromatin pattern, and of nucleoli. Focal or general increase in the density of staining of the nuclei. Vacuoles within the nuclei. Fixed Bouin. Stained H. and E. ×390.

FIG. 7.—Cells 18 hr. after infection. Early changes. Arrows point to eosinophilic intranuclear "inclusions". Fixed alcohol. H. and E. ×919.

FIG. 8.—Uninfected cells fixed with alcohol and stained with acridine orange. The print is made from a colour transparency and the yellow-green (DNA) colour appears white while the red (RNA) colour is only faintly shown. ×720.

FIG. 9.—Infected cynomolgus kidney culture prepared exactly like Fig. 8. There is intense fluorescence around the centre of the nucleus in the cells showing advanced changes. ×720.



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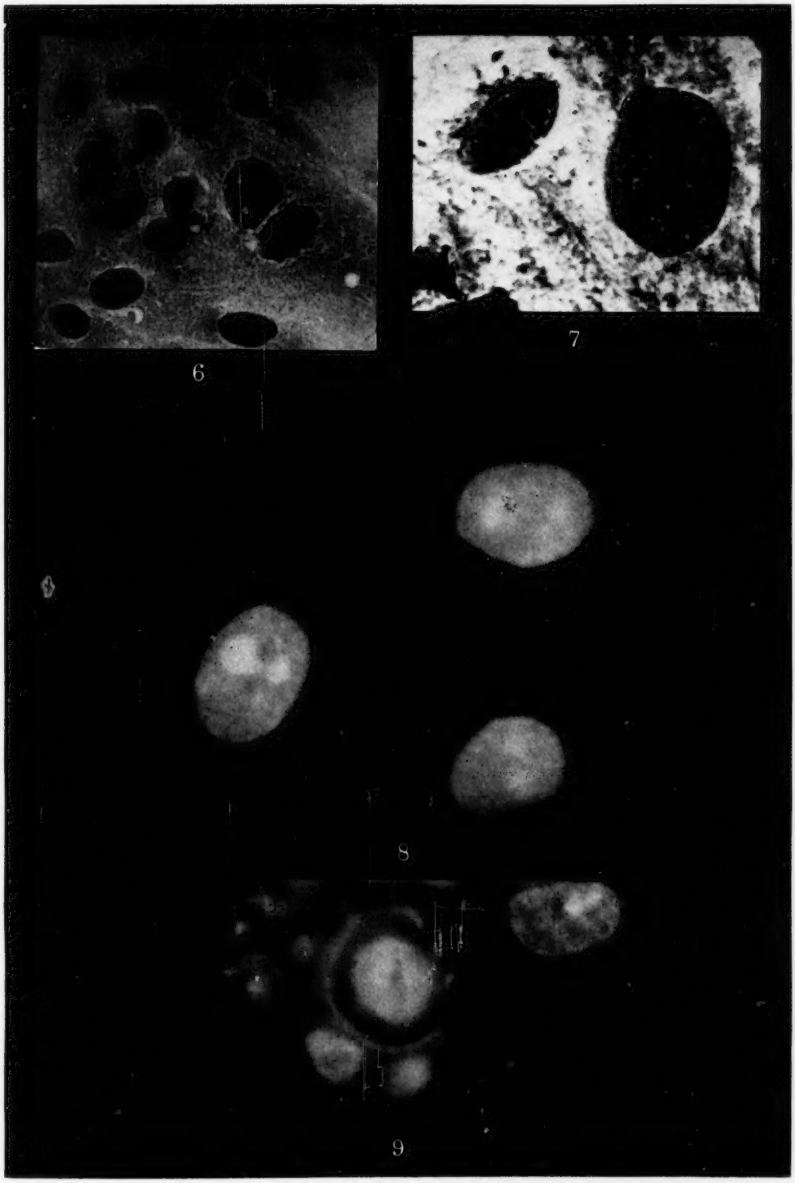

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TOXIC FATTY LIVER IN ADRENALECTOMIZED RATS

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A number of reports in the literature indicate that (total) adrenalectomy prevents the fatty infiltration of the liver which occurs under a variety of treatments or conditions. Laszt and Verzár (1936) reported that adrenalectomy prevents fatty liver produced by phosphorus poisoning in the rat. It was then found by the same authors (Verzár and Laszt, 1936) that, if adrenalectomized rats received adrenocortical extracts together with phosphorus, they developed fatty livers just as did intact rats. Adrenalectomy has also been shown to prevent, or to reduce greatly, the accumulation of fat in the liver which usually occurs after methionine administration (Wool and Goldstein, 1953), fasting (MacKay and Barnes, 1937 ; Leblond, Nguyen-van-Thoai and Segal, 1939), high-fat diets (MacKay, 1937), pancreatectomy (Long, Lukens and Fry, 1936), X-irradiation (Leblond *et al.*, 1939) and partial hepatectomy (Collip, Kutz, Long, Thomson, Toby and Selye 1935 ; MacKay and Carne, 1938 ; Berman, Sylvester, Hay and Selye, 1947 ; Ferrari and Harkness, 1954). According to MacKay (1937), adrenalectomy not only prevents the liver fat deposition ordinarily caused by high-fat diets but, if performed when fat is already accumulated in the liver, it hastens the exodus of fat from this organ. In the experience of several groups of workers (Fry, 1937 ; MacKay and Barnes, 1937 ; Payne, 1949 ; Levin and Farber, 1950), adrenalectomy also prevents the fatty infiltration of the liver after anterior-pituitary extract injections. However, Szego and White (1949) reported an increase of liver fat in both intact and adrenalectomized mice in response to an impure preparation of growth hormone.

Although the influence of adrenal cortex on liver fat is not yet fully understood, the fact that adrenalectomy prevented fatty livers produced by very different treatments appeared to us a datum of great interest. It was thought that, using both intact and adrenalectomized animals, it would have been possible to establish clearly which of the effects seen after a given steatogenic treatment were directly related to the treatment employed and which ones were due to adrenal stimulation. Furthermore, it seemed to us that a comparative study of the biochemical changes produced by steatogenic treatments in intact and adrenalectomized animals would have been very useful to find out which among these changes may have a pathogenic role in the development of fatty livers and which are only the consequence of the accumulation of fat within the cells. With this in mind experiments were planned to study the influence of the adrenalectomy on the effects produced in the liver by 2 typical steatogenic poisons, *i.e.* white phosphorus and carbon tetrachloride. The results both chemical and histological presented here show that, contrary to what is generally accepted, adrenalectomy has very little, if any, influence on the development of fatty livers following repeated injections of phosphorus and CCl_4.

MATERIALS AND METHODS

Male albino rats raised in our own colony and weighing between 150–200 g. were used. They were fed a commercial complete diet (Zoofarm, Padua, Italy) and water *ad lib.* Bilateral adrenalectomy was performed under ether anaesthesia. Adrenalectomized animals were given 0·9 per cent sodium chloride in their drinking water.

Intact and adrenalectomized rats were treated for 5 days with either phosphorus (0·05 ml./100 g. body wt./day of a 0·5 per cent solution of white phosphorus in olive oil, subcutaneously) or CCl_4 (0·2 ml./100 g. body wt./day of a 20 per cent solution of CCl_4 in olive oil, subcutaneously). The treatment of the adrenalectomized animals was begun on the 5th postoperative day. The intoxicated animals, either intact or adrenalectomized, were used 24 hr. after the last injection of the poison ; for adrenalectomized animals this time corresponded to 10 days after the adrenalectomy. Untreated adrenalectomized rats were also used 10 days after the adrenalectomy.

All rats were fasted for the 12 hr. immediately before killing. They were stunned by a blow on the head, bled at the neck and the liver was quickly removed. Portions of liver were placed in 10 per cent formalin for histological study and the remainder of the tissue was dried in an oven at 100° until constant weight. Total lipids were determined by extraction of dry tissue with ethyl-ether-ethanol (3 : 1) in a Soxhlet apparatus and weighing of the fatty residue after removal of the solvent. Paraffin sections were stained with haematoxylin and eosin and frozen sections were stained either with Sudan Black alone or with Sudan III and haematoxylin as a counterstain.

Methods and terminology used in the statistical analysis are those given by Fisher (1950).

RESULTS

Since rats may possess accessory adrenal tissue and there may be strain differences in the amount of this tissue, preliminary experiments were carried out to ascertain the effectiveness of adrenalectomy in producing adrenocortical insufficiency in our strain of rats. All rats recovered readily from the operation, but, if their drinking water was not supplemented with sodium chloride, within a couple of days their condition began to show a progressive deterioration including loss of weight. These animals appeared extremely sensitive to the intoxicating treatment and even if untreated they died in 7–15 days, the mortality being practically 100 per cent. Thus, in all the subsequent experiments drinking water for adrenalectomized animals was supplemented with sodium chloride. Under these conditions the mortality of adrenalectomized animals was very low.

The results of the determinations of liver lipids in both intact and adrenalectomized rats, either untreated or poisoned for 5 days with white phosphorus or CCl_4, are summarized in the Table. It may be seen that, under the present experimental

TABLE.—*Total Lipid Content of Livers from Intact and Adrenalectomized Rats Treated Repeatedly with White Phosphorus and CCl_4 (mg./100 mg. dry Tissue)*

The figures represent the means of 15 determinations ± standard error.

Intact rats treated with			Adrenalectomized rats treated with		
Nil	White phosphorus	CCl_4	*Nil*	White phosphorus	CCl_4
10·0±0·74	25·8±2·60	28·0±2·19	11·8±0·54	22·5±2·16	21·9±1·78

conditions, the lipid content of the liver was practically unchanged in otherwise untreated adrenalectomized animals as compared to normal intact controls. It is also apparent that either phosphorus or CCl_4 caused a great increase of liver lipids

in both intact and adrenalectomized rats. Following either of the poisons, but particularly after CCl_4, the lipid content of the liver appears somewhat lower in adrenalectomized than in intact rats.

A statistical analysis of the results summarized in the Table gives evidence for a highly significant effect of chemical treatments ($P < 0{\cdot}001$) whilst adrenalectomy by itself seems to be of no relevance ($P > 0{\cdot}05$) to the amount of fat in the liver. The effects of the two chemicals used, namely phosphorus and CCl_4, are not quantitatively different ($P > 0{\cdot}05$). The analysis of interaction between adrenalectomy and chemical treatments shows that the over-all effect of chemicals is not significantly influenced by adrenalectomy ($0{\cdot}1 > P > 0{\cdot}05$). However, a more refined analysis testing separately the interaction between adrenalectomy and single poisons indicates that a slight difference in the effects of CCl_4 in intact and adrenalectomized animals cannot be ruled out.

Each liver used for the determination of fat was also examined histologically. The microscopic appearance of the liver of intact rats given repeated injections of phosphorus and CCl_4 is well known and does not need to be described here in detail. For the purposes of the present study it is sufficient to recall that the fat infiltrating the liver after phosphorus intoxication appears almost uniformly distributed throughout the lobules whilst the fatty change occurring after CCl_4-poisoning has a typical mediolobular distribution. As clearly shown by Fig. 1–6, no appreciable difference in the distribution of stainable fat in the liver was found between intact and adrenalectomized rats. The examination of liver paraffin-sections stained with haematoxylin and eosin also did not reveal significant differences between each group of adrenalectomized rats and the corresponding intact controls.

DISCUSSION

That adrenalectomy prevents fatty livers produced by a number of agents, including chemical poisons, is generally accepted as a well-established fact. However, the results both chemical and histological presented in this paper show clearly that adrenalectomy has very little influence, or none at all, in the development of fatty livers following repeated injections of phosphorus or CCl_4 in the rat.

An increase of liver fat 20 hr. after a single dose of CCl_4 by mouth in adrenalectomized rats has been reported recently by Recknagel, Stadler and Litteria (1958). However, the finding that fatty livers after phosphorus poisoning developed in adrenalectomized as well as in intact rats is clearly in contrast with the results of Laszt and Verzár (1936). As mentioned earlier, these authors found that adrenalectomy completely prevented fatty liver after phosphorus poisoning. The difference of results cannot depend upon differences in the intoxication of the animals as both the dosage and the modalities of administration of the poison were exactly the same in our experiments and in those of Laszt and Verzár (1936). On the other hand, although there may be strain differences in the amount of accessory cortical tissue in the rat, there is some evidence that our adrenalectomized rats actually suffered severe adrenocortical insufficiency. It has already been said that the adrenalectomized rats of our stock died in 7–15 days after the adrenalectomy if their drinking water was not supplemented with sodium chloride. The major difference between our experimental conditions and those of Laszt and Verzár (1936) is probably in the amount of sodium taken in by the adrenalectomized animals. Whilst in our experiments drinking water was supplemented

with 0·9 per cent sodium chloride, the fact that Laszt and Verzár (1936) do not state what they gave to drink to their animals makes it likely that their animals were receiving water. On the other hand, the diet used by these authors, containing approximately 1·7 per cent of the McCollum 185 salt-mixture, was not rich in sodium. At present it cannot be said whether the difference in the amount of sodium taken in is responsible for the discrepancy of results; assuming that it is, however, it would follow that the essential for fatty liver to occur after phosphorus poisoning is not the activity of the adrenals but a relative well-being such as can be obtained even in adrenalectomized rats by a high sodium intake. Unfortunately it has not been possible to test this possibility directly since our adrenalectomized rats given water to drink did not survive the treatment with steatogenic poisons. In this connection the observation of Verzár and Laszt (1936) is of interest in that the administration of an adrenocortical extract (not steatogenic by itself under the test conditions) restored in their adrenalectomized rats the ability to develop fatty livers after phosphorus poisoning. This finding is not in contrast with the aforementioned hypothesis, since the cortical-extract therapy is of course the most adequate treatment to maintain the adrenalectomized animals in good condition.

It is apparent that the results presented in this paper do not suggest any clue as to the pathogenesis of toxic fatty liver. It seems worth while, however, to discuss briefly these results in connection with some recent work in the field. It has been shown that in the livers of animals poisoned with phosphorus and CCl_4 there is uncoupling of oxidative phosphorylation (Dianzani, 1954), loss of mitochondrial pyridine nucleotides (Christie and Judah, 1954; Dianzani, 1955), and a lowered content of adenosine triphosphate (Dianzani, 1957). It has also been found that coenzyme A content is decreased in fatty livers produced by phosphorus (Severi-Fonnesu, Fonnesu and Agostini, 1958) or CCl_4 (Garattini, Morpurgo and Passerini, 1955; Heim, Leuschner and Ott, 1956; Severi and Fonnesu, 1956; Severi-Fonnesu *et al.*, 1958).

In a study on the mechanism of action of CCl_4, Christie and Judah (1954) put forward the theory that the poison acted upon liver cells by penetrating the membranes and attacking the mitochondria. Dianzani (1954, 1955, 1957) advanced the hypothesis that the key lesion in toxic (and nutritional) fatty liver was an alteration of the mitochondria leading to a loss of mitochondrial coenzymes and to a reduced supply of adenosine triphosphate. Activation and probably oxidation of fatty acids would be consequently reduced and this could account for the increase in liver fat. As far as CCl_4-treatment is concerned, these views have been challenged by Recknagel and co-workers (Recknagel and Anthony, 1957, 1959; Recknagel, Stadler and Litteria, 1958) on the grounds that after a

EXPLANATION OF PLATE

FIG. 1.—Normal rat liver. Sudan Black. ×80.

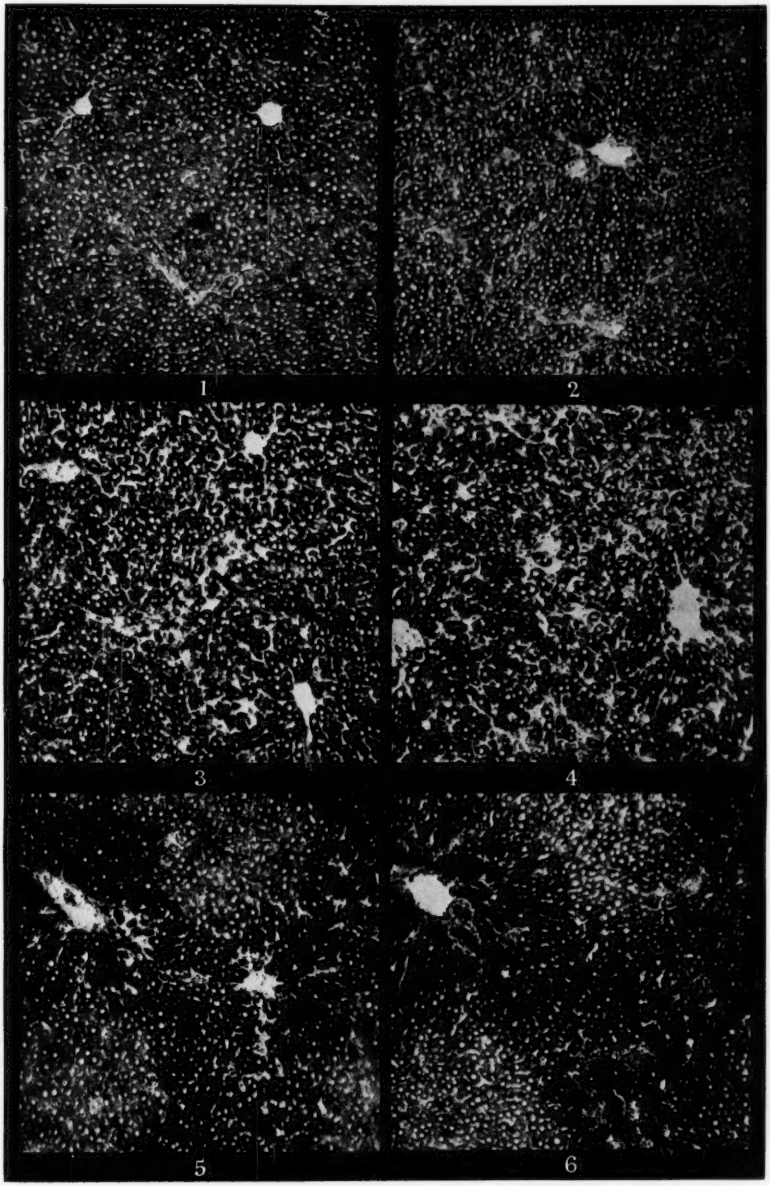
FIG. 2.—Liver from adrenalectomized rat. Sudan Black. ×80.

FIG. 3.—Liver from intact rat treated with white phosphorus. Sudan Black. ×80.

FIG. 4.—Liver from adrenalectomized rat treated with white phosphorus. Sudan Black. ×80.

FIG. 5.—Liver from intact rat treated with CCl_4. Sudan Black. ×80.

FIG. 6.—Liver from adrenalectomized rat treated with CCl_4. Sudan Black. ×80.



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single CCl_4-feeding the alteration of liver mitochondria functions followed the rise in liver fat. Recknagel and Anthony (1959) have suggested that the mitochondrial degeneration and the increase of liver fat arise from two independent lines of pathological development set into motion by an as yet unknown degenerative process which is the primary lesion in CCl_4-hepatotoxicity. It might be thought that the 2 lines were independent even at the origin and that the line leading to the increase of liver fat arose from the stimulation of the adrenals by the steatogenic poison, the mitochondrial degeneration being either independent from, or consequent to, the accumulation of fat within the cells. The observations that fatty livers can be produced by the administration of adrenocortical extracts (Ingle, 1950) and of adrenocorticotrophic hormone (Baker, Ingle, Li and Evans, 1948), in addition to the already mentioned reports indicating that adrenalectomy prevents fatty livers induced by very different treatments, favoured such a view. However, the finding that adrenalectomized rats exhibit an increased liver fat at 20 hr. post CCl_4-feeding (Recknagel *et al.*, 1958) and the present results showing the occurrence of fatty livers in adrenalectomized rats given repeated injections of phosphorus and CCl_4 seem to exclude such a pivotal position of the adrenals in the pathogenesis of toxic fatty liver.

SUMMARY

In view of previous reports indicating that adrenalectomy prevents fatty livers caused by a number of agents in the rat, the influence of adrenalectomy on the effects produced in the liver by 2 typical steatogenic poisons, *i.e.* white phosphorus and CCl_4, has been studied.

Intact and adrenalectomized rats were treated daily for 5 days with either white phosphorus or CCl_4, subcutaneously. The results, both chemical and histological, show that, contrary to what is generally accepted, adrenalectomy has very little, if any, influence on the development of fatty livers after repeated injections of phosphorus or CCl_4.

The results are briefly discussed in connection with recent work on the pathogenesis of toxic fatty liver.

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THE ANTIGENIC RELATIONSHIP BETWEEN PHENOL EXTRACTED BACTERIAL DEOXYRIBONUCLEIC ACID AND OTHER SOLUBLE ANTIGENS OF BRUCELLAE STUDIED WITH THE AID OF THE AGAR GEL PRECIPITATION TECHNIQUE

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THE extraction of deoxyribonucleic acid from *Brucella abortus* with 0·5 per cent phenol was reported by Braun, Burrous and Phillips (1957). On continuation of their work Phillips, Braun and Plescia (1958*a*) reported that the antigenicity of the isolated substance, although resistant to trypsin and trypsin plus chymotrypsin, was altered by deoxyribonuclease. Evidence for the contribution of bacterial DNA to the antigenic specificity of the product was finally obtained by Phillips, Braun and Plescia (1958*b*), who made the following observations: the appearance of Feulgen positive zones among several bands in agar gel diffusion tests; the significant decrease of the precipitability of the DNA preparation after DNase treatment; the occurrence of cross reactions between DNA from calf thymus and salmon sperm and bacterial DNA antiserum which increased with limited depolymerization by DNase. Since according to Olitzki and Sulitzeanu (1958) trypsin and papain had no effect on the stability of most of the antigens described by them (1957), it seemed worthwhile to compare them with the products obtained by Braun *et al.* (1957) to ascertain whether or not these products obtained by different methods were identical.

The general plan of the work was as follows: precipitation tests with products obtained from brucellae by disintegration as well as by extraction with phenol were performed. Attention was paid to the position and continuity of the bands obtained by the reaction of both bacterial products with the same antisera. Absorption tests with both products and the examination of their remaining antibodies were carried out for the purpose of identification. The sensitivity of both products to the action of DNase and the antigenicity resulting from this treatment were examined.

MATERIAL AND METHODS

The immune sera were the same as those employed by Olitzki (1959). The disintegration of the bacteria was carried out in a disintegrator (Mickle, Mill Works, Gomshall, Surrey). The resulting bacterial extracts were freed from insoluble particles by centrifugation at 10,000 r.p.m. for 30 min. Some DNA-preparations were prepared using the technique described by Braun *et al.*, (1957), and others were kindly provided by Dr. W. Braun, Institute of Microbiology, Rutgers University (The State University, New Brunswick, New Jersey). The diffusion tests were carried out according to the technique employed by Olitzki (1959).

RESULTS

Position and shape of the produced precipitation bands

Fig. 1 shows the results obtained with 3 DNA-protein preparations, one of which was prepared by the author from *Br. melitensis*, the other 2, provided by Dr. W. Braun, were prepared from *Br. suis* and *Br. abortus*. All 3 antigens, although derived from different strains in different laboratories, produced the same sharp line close to the serum source. The product of *Br. melitensis* produced 2 additional lines. Fig. 2 shows the serological differences between the products obtained in the course of the preparation of the DNA-protein. The extracted bacteria produced only some diffuse bands, one of them, probably antigen 6, common to the proteins precipitated with chloroform and amyl alcohol and to the DNA-protein. In addition, the DNA-protein produced 2 lines which were not shared by anyone of the resulting products. Fig. 3 shows that not all antisera yielded the same type of band when they reacted with the same antigen. Three different *Br. melitensis* antisera reacted with the homologous *Br. melitensis* DNA-protein to produce a line which was similar in position and shape to line 6 obtained with disintegrated bacteria, but only 2 of them produced an additional sharp line similar to line 5 obtained with disintegrated bacteria. Serum C produced a 3rd line corresponding to the position of line 4.

Experiments with constant amounts of immune sera and graded amounts of DNA-protein

Table I shows that the number of lines produced varied directly with the amounts of antigen (DNA-proteins) used in the reaction with the undiluted

TABLE I.—*Production of Precipitation Lines by Graded Amounts of DNA-proteins Reacting with* 0·1 *ml. of Undiluted Antiserum*

DNA derived from	Reacting amounts (μg.)	Number of lines produced with antisera prepared by immunization with			
		Br. melitensis (6015)	*Br. melitensis* (Rev. I)	*Br. suis* (39)	*Br. abortus* (2308)
Br. melitensis (6015)	1000	3	2	2	2
	500	3	2	2	2
	250	3	1	2	1
	120	2	1	1	1
	60	1	1	1	1
	30	1	1	1	1
	15	0	0	0	0
Br. suis (39)	1000	3	2	2	2
	500	3	2	2	2
	250	3	2	2	2
	120	2	1	1	1
	60	1	0	1	0
	30	0	0	1	0
	15	0	0	0	0
Br. abortus (2308)	1000	4	3	2	2
	500	3	3	1	1
	250	2	2	1	1
	120	2	1	1	1
	60	1	1	1	1
	30	1	1	0	1
	15	0	0	0	1
	7·5	0	0	0	0

antisera. Large amounts of all DNA-proteins gave several precipitation lines. The number of lines decreased with a decrease in antigen. And small amounts less than 100 μg. yielded no more than one line, which in most cases was thin, sharply defined and close to the serum source, similar to line 5 obtained with bacterial extracts. No conclusion as to specificity could be drawn in experiments where preparations were employed in minimal amounts. The product derived from *Br. melitensis* gave visible reactions with all antisera, when amounts of 30 μg. were present. The products of *Br. suis* and *Br. abortus* required lesser amounts of antigen (30 and 15 μg., respectively) for a visible reaction with their homologous antisera than with heterologous antisera. The disappearance of the multiplicity of bands with the decrease of antigen concentration was common to all 3 antigens, which, applied in minimal reacting doses, produced only one band similar in shape and position to band 5 of disintegrated bacteria.

Experiments with constant amounts of antigens and varying dilutions of immune sera

Table II shows the results obtained when DNA-proteins reacted with varying dilutions of antisera. As shown by Olitzki (1959) all Brucella antisera when diluted to 1 : 20 or more retained their ability to produce line 5 with disintegrated bacteria.

TABLE II.—*Production of Precipitation Lines by Graded Dilutions of Antibacterial Immune Sera Reacting with* 0·1 *mg. of DNA-protein*

Antisera prepared by immunization with:	Reciprocals of serum dilutions	Precipitation lines produced with DNA-protein derived from		
		Br. melitensis (6015)	*Br. suis* (39)	*Br. abortus* (2308)
Br. melitensis (6015)	1	2	2	2
	2	2	2	2
	4	1	2	1
	8	1	1	1
	16	1	1	1
	32	0	0	1
	64	0	0	0
Br. melitensis (Rev. I)	1	3	1	3
	2	2	1	2
	4	2	1	2
	8	2	1	2
	16	2	1	1
	32	1	1	1
	64	0	0	0
Br. abortus (2308)	1	2	1	2
	2	1	1	2
	4	1	0	1
	8	0	0	0
Br. suis (39)	1	1	1	1
	2	1	1	1
	4	0	1	1
	8	0	1	0
	16	0	0	0
Br. suis (39)	1	1	1	1
	2	1	1	1
	4	1	1	1
	8	1	1	1
	16	1	1	0
	32	0	1	0
	64	0	0	0

Table II shows that a reaction is also produced when DNA-proteins combine with serum even as dilute as 1 : 32. The position and shape of the resulting band was similar to that of line 5 obtained with disintegrated bacteria. The titres of the sera depended entirely on their strength and no indication for a strain specificity of their antibodies was found. Two *Br. melitensis* and one *Br. suis* antisera gave positive reactions at dilutions of 1 : 16 and 1 : 32 with all antigens, while another *Br. suis* and *Br. abortus* antisera gave visible reactions only at higher serum concentrations.

Figs. 4 and 5 illustrate the results obtained with DNA-protein derived from *Br. suis* and are presented in Table II. Fig. 4 represents the experiment with the homologous immune serum. Only one sharp line was produced. In Fig. 5 the experiment with the *Br. melitensis* immune serum is presented. In the stronger concentrations in addition to the sharp precipitation band a 2nd more diffuse band appeared close to the serum source. At the higher serum dilutions this additional band disappeared, but the sharp band persisted.

Attempts to identify the DNA band with one of the bands produced using sonic extracts

Fig. 6 illustrates an experiment in which disintegration extracts of *Br. melitensis* and *Br. abortus* and DNA-protein of *Br. melitensis* reacted simultaneously with the same *Br. suis* immune serum. Opposite SEM there appeared 2 strong bands, 6 and 5, and 2 weaker ones, 3 and 2. Band 6 became split into 2 partial lines. Opposite SEA an additional line, 4, was also visible. Lines 3 and 2 were stronger opposite SEA than opposite SEM. The precipitation bands produced by DNAM are generally weaker with line 6 hardly visible. It is clear that the only persisting line produced by the DNA-protein is the continuation of line 5 produced by the sonic extracts.

These results were confirmed by absorption experiments. Table III shows the precipitation lines given by *Br. melitensis* immune serum (diluted 1 : 4) after

TABLE III.—*Absorption Experiment with* Br. melitensis *Antiserum*

Dilution of immune serum	Absorption with 2·5 mg. DNA of	Precipitation lines produced by the following antigens: DNA *Br. abortus*	DNA *Br. suis*	DNA *Br. melitensis*	*Br. melitensis* disintegrated bacteria
1 : 4	*Br. abortus*	—	—	—	5
	Br. suis	—	—	—	5
	Br. melitensis	—	—	—	5
1 : 8	*Br. abortus*	—	—	—	—
	Br. suis	—	—	—	—
	Br. melitensis	—	—	—	—
1 : 4	Non absorbed	5·6	5·6	5·6	5·6*
	Non absorbed	—	—	—	5*

* Disintegrated *Br. abortus* and *Br. suis* produced the same lines.

absorption with 2·5 mg. DNA-protein prepared from any Brucella strain. No precipitation bands were produced by this serum with any of the 3 DNA-proteins while line 5 persisted with all 3 sonic extracts. However, when the same experiment

was repeated with the same immune serum diluted 1 : 8 the precipitation reaction with the sonic extracts also became negative. We repeated this experiment with a strong *Br. suis* immune serum and with larger quantities of absorbing antigens, in order to be certain that these results are not due to an additional component of antigen 5 present only in the disintegration extracts but to quantitative differences. Table IV shows that after absorption with quantities of DNA-protein as large as

TABLE IV.—*Absorption Experiment with* Br. suis *Antisera*

Dilution of immune serum	Absorption with DNA *Br. melitensis*	Lines which appeared with: DNA *Br. melitensis*	DNA *Br. abortus*	DNA *Br. suis*	Extract *Br. melitensis*	Extract *Br. suis*
1 : 2	5 mg.	5·6	—	5·6	5	5
1 : 5	8 mg.	—	—	—	5	5
1 : 10	9 mg.	—	—	—	5	5
1 : 2	Non absorbed	5·6	5·6	5·6	5·6	5·6
1 : 5	,, ,,	5	5	5	5	5·6
1 : 10	,, ,,	—	—	—	5	5·6

9·0 mg. precipitins for the bacterial disintegration extracts persisted while those for the DNA-protein had already disappeared after absorption with 8·0 mg. In addition, the non-absorbed immune serum was still positive for the bacterial extracts at a 1 : 10 dilution but negative for the DNA-proteins at this dilution.

The sensitivity of soluble Brucella antigens to DNase

2·5 mg. of DNA-protein were dissolved in 5 ml. of physiological saline. To this solution 0·5 ml. of a 0·1 M $MgSO_4$-solution and 1 mg. of crystallized DNase (Worthington) were added. Bacterial extracts were prepared by disintegration of bacteria as described by Olitzki (1959). The extracts were freed of insoluble particles by centrifugation at 10,000 r.p.m. To 5 ml. portions of the clear supernatant fluids the same quantities of $MgSO_4$ and DNase were added, as to the DNA-protein solutions. After the mixtures were incubated at 37° for various intervals of time starting with 1 hr., their antigenic properties were examined by the agar gel precipitation test. Whereas the DNA-proteins lost their antigenic properties after 1 hr. exposure to DNase those of the bacterial extract were preserved even after 24 hr. incubation.

Production of anti-DNA-protein immune sera

In addition to the antibacterial immune sera employed in the above described experiments other immune sera were produced by immunization of rabbits with DNA-proteins. These antigens were suspended in an adjuvant mixture as described by Olitzki (1959). One mixture contained 1·25 mg. of *Br. melitensis* DNA-protein and the other 0·4 mg. of *Br. abortus* DNA-protein per ml., respectively. Six intramuscular injections were given at 8-day intervals. The total amount of *Br. melitensis* DNA-protein injected in the course of the immunization was 10 mg. and that of *Br. abortus* 3 mg. Twelve days after the last injection blood was obtained by cardiac puncture. In the rabbits immunized with *Br. abortus*-DNA-protein the immunization was continued using a mixture which contained 7·5 mg. per ml. of the same antigen. Four subcutaneous injections of increasing doses were given

at 7-day intervals and a total of 37·5 mg. DNA-protein were administered. The immune sera of the animals taken after this additional treatment did not show any antibody pattern different from those obtained with smaller quantities, 3·0 and 10·0 mg. of DNA-protein, respectively.

Precipitation experiments with anti-DNA-protein immune sera

The immune sera were brought into contact with sonic extracts of various brucella strains. Even when undiluted they did not produce more than 3–4 precipitation lines. In this respect they differed from the antibacterial immune sera which produced 5–7 lines (Olitzki, 1959). In dilutions greater than 1 : 4 one sharp precipitation line was produced which according to its position and shape was similar to line 5, as represented in Table V. With DNA-proteins of virulent

TABLE V.—*Production of Precipitation Lines by Graded Dilutions of Anti-DNA-protein Immune Sera Reacting with Extracts of Disintegrated Bacteria*

Antisera prepared by immunization with DNA-protein of	Reciprocals of serum dilutions	Precipitation lines produced with bacterial extracts of		
		Br. abortus 19	*Br. suis*	*Br. melitensis* 5
Br. abortus 2308	1	4	3	3
	2	2	3	3
	4	2	3	2
	8	1	1	2
	10	1	1	1
	32	1	1	1
	64	0	1	0
Br. melitensis 5	1	4	2	4
	2	2	2	4
	4	2	2	3
	8	1	1	1
	10	1	1	1
	32	1	1	0
	64	0	0	0

and non-virulent Brucellae these sera rarely produced more than one precipitation line ; this line appeared even with serum dilutions up to 1 : 64, as seen in Table VI. No significant differences were observed in the precipitating ability of DNA-proteins prepared from different species nor in the DNA-proteins from virulent and non-virulent strains of the same species, *e.g. Br. abortus* strains 2308, 19 and 19D.

The antigenic identity of all these DNA-proteins was proved by mutual absorption of their corresponding antibodies from homologous and heterologous immune-sera and by agar diffusion tests : when placed around the same central serum source as in the experiment reproduced in Fig. 1, all these DNA-proteins produced the same continuous ring-shaped line.

In addition to the precipitins the DNA-immune sera contained agglutinins for all Brucella strains with titres of 1 : 2000 to 1 : 5000. It seems that these agglutinins were produced by small amounts of cell wall antigens which were present in the DNA-preparations. Chemical examination showed that some of the preparations contained about 0·3 per cent diaminopimelic acid, indicating the presence

TABLE VI.—*Production of Precipitation Lines by Graded Dilutions of Anti-DNA-protein Immune Sera Reacting with* 1·0 *mg. of DNA-protein*

DNA proteins derived from	Reciprocals of serum dilutions	Number of precipitation lines produced with antisera prepared by immunization with DNA of	
		Br. abortus 2308	*Br. melitensis* 5
Br. abortus 19	1	2	1
	2	1	1
	32	1	1
	64	0	0
Br. abortus 19 D	1	1	1
	16	1	1
	32	0	1
	64	0	0
Br. abortus 2308	1	2	1
	4	2	1
	8	1	1
	32	1	1
	64	0	0
Br. melitensis 5	1	1	1
	8	1	1
	64	1	1
	128	0	0
Br. suis	1	1	1
	32	1	1
	64	1	0
	128	0	0

of cell wall substances. After absorption with DNA-proteins the precipitating power of the DNA-immune sera disappeared, while their agglutinating power was still intact. On the other hand, by absorption with acetone treated bacteria the agglutinin titre decreased 100 times, while the precipitation power still remained intact. These experiments proved that the DNA-proteins themselves were not agglutinogenic, but contained small amounts of agglutinogenic cell wall substance.

The relationship between DNA-proteins and antigen 5 *tested by anti-DNA-protein immune sera*

Fig. 7 shows the results of a typical agar diffusion test with a *Br. abortus* DNA-protein immune serum. Various bacterial products of the homologous strain, which were obtained in the course of the extraction and purification of the DNA-protein, were placed around the source of the antibody. These products were : the insoluble bacterial substance remaining after 10 extractions with 0·5 per cent phenol, the proteins which were precipitated with amyl alcohol and chloroform, and the purified DNA-protein itself. The precipitating potency of a sonic extract of the same bacterial strain was compared with that of the above mentioned products. Unfortunately, it was impossible to obtain a photograph showing all important details. A drawing was, therefore, prepared in which all the precipitation lines obtained in the conclusive experiment were shown schematically.

The figure shows that (1) the 10 times extracted bacteria did not produce any

precipitation line, (2) that the precipitated bacterial protein shares a line with the sonic extract very close to the serum source, probably line 6 of sonic extracts, and (3) that the DNA-proteins and the sonic extracts share an antigenic component which produces a line situated at the same distance from the serum source as line 5. However, this antigen is not completely identical with antigen 5 of the sonic extract. Fig. 7 shows clearly that in addition to the antigen shared by the DNA-protein and the sonic extract, there exists a second component. The line produced by both components is seen between the source of the immune serum and the sources of the sonic extracts. Part of it becomes bent and continues to run between the source of the immune serum and the sources of the DNA-proteins. Another part of the band, mainly that on the right side of the figure, runs as a straight line between the source of the sonic extract and the source of the immune serum and ends just at the edges of the DNA-protein sources, indicating that this antigen is not shared by the DNA-protein.

Similar results were also obtained with the *Br. melitensis* DNA-protein immune serum. These experiments showed that antigen 5 of the sonic extracts contained a component shared by the DNA-proteins but was not identical with them.

DISCUSSION

The experiments described above show that by extraction with phenol and further purification DNA-proteins were isolated, which were antigenically identical to those isolated by Braun *et al.* (1957). It was not possible to differentiate these antigens by serological methods. Their corresponding antibodies were removed by absorption from immune-sera irrespective of whether their origin was from *Br. abortus*, *Br. melitensis* or *Br. suis*. The precipitation line common to all 3 antigens was situated close to the serum source in the same zone as band 5 which was produced by bacterial extracts obtained either by mechanical disintegration or sonic vibration. However, it cannot be definitely stated whether or not the DNA-proteins are identical with antigen 5. The following data are not consistent with the assumption that the 2 substances are identical.

The DNA-proteins had a relatively low combining power. In absorption experiments they were only able to remove the antibodies reacting with themselves often leaving in the immune serum antibodies which reacted with bacterial sonic extracts and produced line 5.

The behaviour of the DNA-proteins was different from that of antigen 5 towards DNase. It was possible to destroy with the aid of this enzyme the precipitability of some DNA-proteins obtained by phenol extraction, but not that of antigen 5 present in the sonic extracts. DNase was able to destroy the precipitability of some of the DNA-proteins, obtained by phenol extraction, but not that of antigen 5 present in sonic extracts.

In certain precipitation experiments as that shown in Fig. 7 it was observed that band 5 contained one component which was identical and another which was not identical with the DNA-proteins.

It seems, therefore, that antigen 5 is associated in sonic extracts with a component which controls its antigenic specificity. This component does not seem to be a protein since according to Olitzki and Sulitzeanu (1958) antigen 5 was not destroyed either by trypsin or papain, but was inactivated by treatment with 0·1 N HCl for 24 hr. at 20°. Braun, Plescia and Palczuk (1959) after comparing

the physico-chemical alterations of their phenol-extracted DNA-proteins with their antigenic reactivity after DNase-treatment observed that some batches showed physico-chemical and antigenic alterations, others showed physico-chemical alterations without any loss of antigenic reactivity and in still others both characteristics remained unchanged. They, therefore, suggested that there are at least 3 types of antigens : (1) those in which antigen-specificity is controlled by DNA ; (2) those in which DNA is associated with antigens of a different chemical nature, and (3) DNA-free antigens. Their second suggestion is supported by the observations of Stahl, Pennel and Huddleson (1939), which state that the serological activity of protein-nucleates from S-strains is associated with the protein, and not with the nucleic acid part of the fraction. On the other hand, Topping (1934) isolated nucleoproteins from *Br. abortus* and *Br. melitensis* by extraction with 0·01 N NaOH and precipitation with 10 per cent acetic acid. These products were non-species-specific and reacted with *Br. abortus* and *Br. melitensis* immune sera like the DNA-proteins described in this paper. However, they contained carbohydrates which were non-species-specific too and produced with Brucella immune-sera precipitates. These definite precipitation reactions obtained with Brucella immune-sera suggested that the carbohydrate was a pre-existing antigenic element of the bacterial cells and was not derived from the cell nucleoproteins by hydrolysis.

The presence of agglutinogenic substances in batches of DNA-proteins prepared by extraction with phenol was to be expected in spite of further purification procedures in view of the fact that the extraction of brucellae by phenol as first carried out by Miles and Pirie (1939) led to the isolation of the antigenic (PLAPS)-complex, which interfered with the reaction of agglutinins with intact bacteria.

SUMMARY

DNA-conjugated proteins isolated from *Br. abortus*, *Br. melitensis* and *Br. suis* were highly antigenic and when injected into rabbits produced precipitating immune sera. These immune sera reacted with the antigens in agar diffusion experiments producing one sharp precipitation line up to a dilution of 1 : 64. They gave a positive flocculation test in tubes up to a dilution of 1 : 128. No DNA-conjugated protein specific for a single species of the Brucella genus was demonstrated among these antigens.

The production of bacterial agglutinins by DNA-proteins is ascribed to the presence of small amounts of agglutinogens in the preparation. These agglutinins were removed by intact bacteria, leaving the precipitins reacting with DNA-conjugated proteins intact.

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EXPLANATION OF PLATE

FIG. 1.—Diffusion pattern of 3 DNA-proteins reacting with undiluted *Br. suis* antiserum.

I.S.Suis = *Br. suis* immune serum.
DNAM = DNA-protein prepared from *Br. melitensis*.
DNAS = DNA-protein prepared from *Br. suis*.
DNAA = DNA-protein prepared from *Br. abortus*.

FIG. 2.—Diffusion pattern of DNA-protein, simple protein and bacteriae extracted by phenol reacting with uniluted *Br. suis* immune serum.

S = *Br. suis* immune serum.
1 = *Br. melitensis*-bacteria extracted by phenol.
2 = *Br. melitensis*-protein removed from phenol extract by chloroform and amyl alcohol.
3 = *Br. melitensis*-DNA-protein.
4 = *Br. suis*-bacteria extracted by phenol.

FIG. 3.—Diffusion pattern of DNA-protein prepared from *Br. melitensis* with three different *Br. melitensis* immune sera.

DNAM = DNA-protein prepared from *Br. melitensis*.
A, B and C = Three *Br. melitensis* immune sera.

FIG. 4.—DNA-protein derived from *Br. suis* reacting with varying dilutions of *Br. suis*-immune serum.

DNAS = DNA-protein derived from *Br. suis*.
1 : 4, 1 : 8 etc. = Serum dilutions.

FIG. 5.—DNA-protein derived from *Br. suis* reacting with varying dilutions of *Br. melitensis* immune serum.

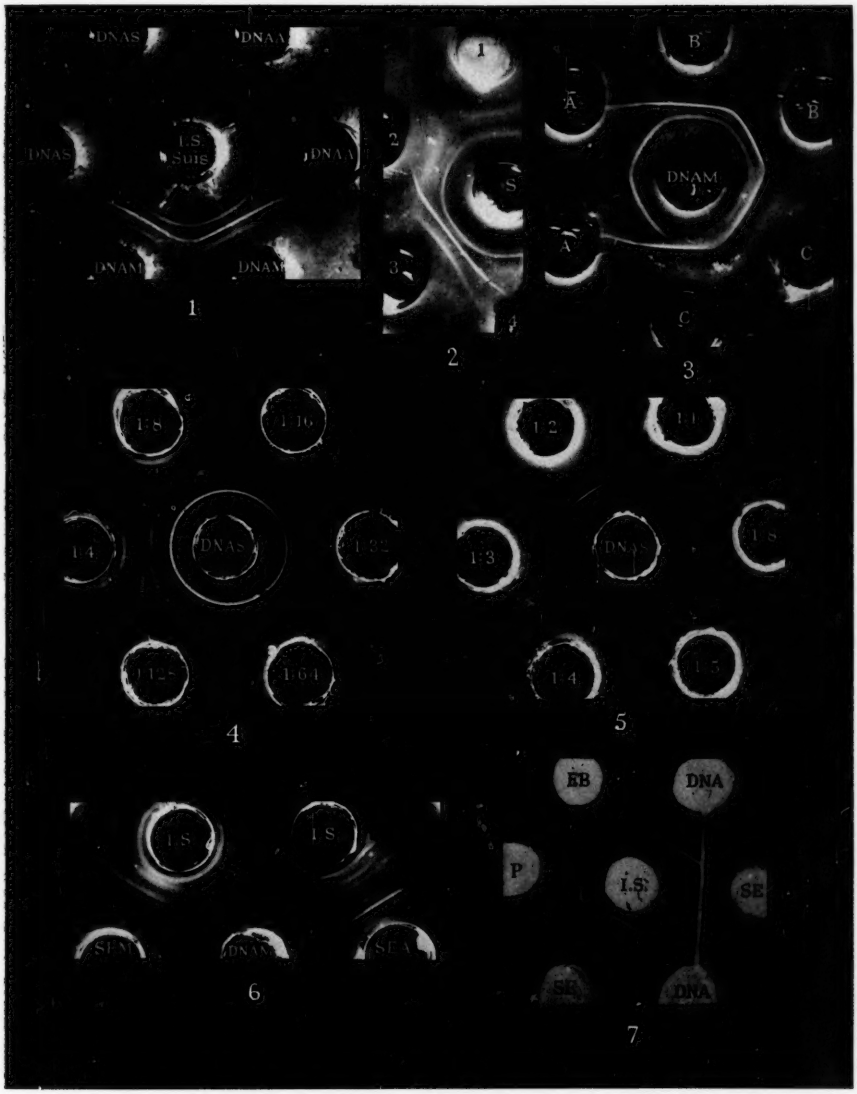
DNAS = DNA-protein derived from *Br. suis*.
1 : 1, 1 : 2, 1 : 3, etc. = Serum dilutions.

FIG. 6.—Attempt to identify the DNA-band with one of the bands produced by sonic extracts.

I.S. = *Br. suis*-immune serum.
SEM = Supernatant fluid of extract prepared from *Br. melitensis*.
SEA = Supernatant fluid of extract prepared from *Br. abortus*.
DNAM = DNA-protein prepared from *Br. melitensis*.

FIG. 7.—Diffusion pattern of *Br. abortus* DNA-protein immune serum with different fractions derived from the same strain.

I.S. = Immune serum.
DNA = DNA-protein.
SE = Sonic extract.
P = Protein precipitated by amyl alcohol and chloroform.
EB = Bacteria extracted × 10 with 0·5 per cent phenol.


DNAS
DNAA
LS
SUIS
DNAM
1
2
3
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6
7
EB
DNA
P
SE

THE ELECTROCARDIOGRAPHIC APPEARANCES OF MYOCARDIAL INFARCTION IN THE RAT

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This report is concerned with electrocardiographic investigations made on rats subjected to procedures designed to produce myocardial infarction by dietary means (Thomas and Hartroft, 1959) or by injections of isoproterenol (Rona, Chappel, Balazs and Gaudry, 1959). Such electrocardiograms provide early evidence of infarction in the living animal.

An attempt has also been made to define the normal electrocardiogram of the rat.

METHODS

Electrocardiograms.—Unanaesthetized rats were secured in a clamp (Fig. 1) described by Drury, Harris and Maudsley (1930), care being taken to avoid pressure on the neck. Electrodes, which consisted of small hypodermic (BD20) needles, were inserted under the skin of the left and right forelimbs and the left hindlimb. The leads were connected to a Matthew's portable electrocardiograph of the moving mirror type manufactured by the Clifton Instrument Co. Cambridge. Standard leads were taken in the conventional manner. When taking a precordial lead, the leads from the limbs were each connected through a 5000 ohm resistance ; the precordial electrode was inserted under the skin at points on the thorax to correspond to the conventional positions in man (V_{1-6}) and connected to the instrument with the switch set to the standard lead 1 position.

A few electrocardiograms were taken for comparison using a direct writing instrument (" Electrite model ", Cambridge Instrument Company, Cambridge).

Diets.—Laboratory bred piebald rats were fed a stock diet after weaning and used as normal controls. After reaching 100 g. body weight, 25 male rats were also fed a diet known to produce myocardial infarction (Thomas and Hartroft, 1959) consisting of cholesterol 50g., thiouracil 3 g., cholic acid 20 g., butter 400 g., sucrose 169 g., casein 200 g., choline chloride 10 g., salts 40 g., cellulose powder 100 g., magnesium oxide 5 g., inositol 2 g., thiamine 16 mg., riboflavin 16 mg., pyridoxine HCl 16 mg., folic acid 10 mg., calcium pantothenate 40 mg., biotin 0·6 mg., B_{12} 0·05 mg., nicotinamide 0·2 g.; a weekly supplement comprising 1000 i.u. vitamin A and 40 i.u. vitamin D supplied as one drop of halibut liver oil, 2 mg. α-tocopheryl acetate in 2 drops arachis oil and 0·05 mg. 2-methyl-1 : 4 naphthoquinone in one drop arachis oil.

Injections of isoproterenol.—Isoproterenol (1-[3,4 dihydroxyphenyl]-2 isopropylamino ethanol) was injected as the sulphate (Abbott's Laboratories Ltd.) or hydrochloride (supplied by Dr. G. Rona, Research Laboratories, Ayerst, McKenna and Harrison, Ltd., Montreal) in doses ranging from 5–80 mg. free base/kg. body weight. They were given subcutaneously and intraperitoneally to normal rats of both sexes ranging from 100–300 g. body weight. The minimum period of injection was 2 consecutive days but some animals were injected daily for several weeks.

Animals fed a diet known to produce myocardial infarction survived for an average period of 100 days. Necropsy was carried out as soon as possible after death. Rats injected with isoproterenol and normal controls survived and were killed by ether anaesthesia. Specimens were taken of the heart, thoracic and abdominal aorta and fixed in neutral buffered formal saline. Frozen sections were stained by Oil red O and paraffin sections by a variety of conventional methods.

RESULTS

Normal animals

The normal rat electrocardiogram showed P, Q, R, S and T waves as in man. Of the 3 standard limb leads, lead 2 appeared to show the individual waves best. Precordial leads V_1 and V_2 (and sometimes V_4) were recorded.

A conspicuous feature was elevation of the S-T segment in all leads examined (Fig. 2*a* and 2*b*) : this was an almost invariable finding and made the normal rat electrocardiogram bear a superficial resemblance to that of myocardial infarction or pericarditis in man. Lombard (1952) noted the lack of an iso-electric S-T segment in the rat and other small mammals, with the exception of the guinea pig.

Q waves, when they could be distinguished, were always small ; S waves were found infrequently. An N wave has been described in the rat electrocardiogram by Heise and Kimbel (1955). We could find no clear evidence of this positive deflection between QRS and T, either with photographic or direct writing electrocardiogram methods, but occasionally the T wave showed a double peak.

Precordial leads showed upright P waves followed by ventricular complexes with upright T waves. There was little change in the electrocardiogram as the chest lead position was moved across the precordium ; the main deflection was the R wave, which was closely followed by T, with a shallow trough of S–T segment between. The voltage of Rv_1 was usually lower than that of Rv_2–v_4. In lead V_1, T was as tall as, or taller than R. In leads V_2 and V_4, T was nearly always lower than R. S waves were seen rarely, and only in lead V_1.

The heart rate usually lay between 380–480/min. and abnormal rhythms were not seen. The important observation was made that pressure on the neck by the neck clamp could cause a marked reduction in heart rate : in one case from 450/min. to 150/min., with the appearance of sinus arrhythmia (Fig. 3*a* and 3*b*). Another animal, which had a normal heart at necropsy responded with a prolonged P–R interval, ectopic and dropped beats (Fig. 4). In man, A–V block of this degree indicates organic heart disease.

The heart rate was often so fast that P waves were partly superimposed on antecedent T waves, preventing accurate measurement of the P–R and Q–T intervals. Where they could be measured the normal range was found to be P–R 0·04–0·06 sec. and Q–T 0·08–0·10 sec. Where an S wave was present the QRS interval was found to be in the region of 0·02 sec.

Electrocardiograms were carried out over a period of 4 months in 10 normal rats to look for the effects of increasing age. During this interval there was a fall in heart rate associated with lowering of the T wave, the peak tending to become rounded or slurred. T wave inversion was not seen. The average height of R in lead V_1 fell from 4·5 mm. to 1·7 mm.

Animals on " infarct " producing diet

Serial electrocardiograms of the 25 animals in this group were examined before the results of necropsy were known, and 5 animals were thought to show clearly abnormal tracings. Reference to the histological reports revealed that 4 of these 5 had myocardial infarction ; the mechanism of infarction is discussed elsewhere (Gresham and Howard, 1960). The animal which did not show cardiac infarction at necropsy nevertheless had a ball thrombus in the aorta which may have partially occluded the coronary ostia (Fig. 5).

One animal with histological evidence of an infarct had normal electrocardiograms, the last tracing having been taken 12 days before death. The histological appearances in the heart were those of recent infarction ; probably not more than a week old.

One animal developed a prominent Q wave in lead V_1 (Fig. 6) with upright T in lead V_1 and normal leads 2 and V_2. It died 21 days later and had a transmural infarct.

Two animals developed inverted T waves, and were found at necropsy to have subendocardial infarction. The abnormalities appeared first at 39 and 15 days before death. One of these animals had inverted T waves in leads 2 and V_2 ; in the other one the inverted T was limited to lead V_1. These abnormalities persisted in all later electrocardiograms being seen in one animal's tracings over 34 days. In these 2 animals, T inversion was complete, negative T waves (Fig. 7 ; Fig. 8) following R with little to indicate an S–T segment between them. However one of them showed partial, or terminal, T inversion in V_2 at first (Fig. 9) ; complete T inversion appeared later and was associated with a fall in R amplitude from 3 to 1 mm.

One animal developed sinus bradycardia of 100/min. and terminal inversion of T in lead V_1 only. It died next day and was found to have an apical infarct.

None of the 21 animals on the diet which had normal hearts at necropsy showed T wave inversion of either kind.

The only arrhythmia seen was caused by two probable premature ventricular contractions, in an animal with massive infarction and inverted T waves. (Fig. 10.)

Animals injected with isoproterenol

Electrocardiograms were taken of 8 animals being given isoproterenol. Two animals, given 80 and 40 mg. free base/kg. body weight by daily injection, showed sinus tachycardia of 450—500/min. in tracings taken just after injection on the second day ; both had partial T wave inversion in V_2 and V_4.

Two other animals, given 20 mg. free base/kg. by daily injection showed tachycardia of the same order 24 hr. after the second injection ; one had partial T wave inversion in V_2 only, the other lowering and slurring of T in V_2 without inversion in any lead.

Electrocardiograms of 4 further animals were taken on the day they were killed, 24 hr. after the last injection (25 mg. free base/kg.). The heart rate was 330–370/min. and only one animal had T inversion—partial inversion in V_2 associated with a rate of 370/min. The other 3 animals had varying degrees of T wave slurring.

At necropsy, although the hearts were macroscopically normal, all of the animals had small foci of subendocardial necrosis, mainly in the left ventricle. The coronary arteries were normal.

DISCUSSION

The normal electrocardiograms were similar to those described by Lombard (1952), Irmak and Aykut (1955) and Heise and Kimbel (1955), but the N wave described by the latter workers was not detected using both direct writing and photographic methods. Heise and Kimbel worked with anaesthetised animals, which reduced tachycardia, and used a faster paper speed (80 mm./sec. as compared with 30 mm./sec.), factors which may account for the difference.

Elevation of the S–T segment, which was found constantly in the precordial leads, was a normal feature. Lombard (1952) has observed this in the rat and other small mammals. The reason for this difference from the normal electrocardiogram in man is not known. Lowering of the T wave, with rounding or slurring of the peak, was found in normal animals with increasing age, but T inversion was not found in lead 2 or in the precordial leads.

In the animals fed on infarct producing diet, T wave inversion was associated with myocardial infarction in all but one animal, and this animal died with a ball thrombus in the aorta. Its electrocardiograms showed constant T wave inversion over 17 days and then reverted to normal on the day before death. It is likely that the thrombus partially or intermittently occluded the coronary ostia, resulting in myocardial ischaemia. Our findings suggested that T inversion indicated myocardial ischaemia, and that ischaemia usually led to subendocardial infarction without further change in the electrocardiogram. Certainly in man the transient ECG changes of myocardial ischaemia are indistinguishable from those of subendocardial infarction (Goldberger, 1953). Although S–T depression is characteristic of these conditions in man, no depression was seen in the rat ; it might however have been masked by the normal elevation of S–T. No evidence was found of abnormal S–T elevation comparable with that seen in man after coronary occlusion. One animal with transmural infarction showed a prominent Q wave in V_1 21 days before death. This may be comparable with the abnormal Q wave of human transmural infarction, but other animals were found with RS patterns in lead V_1 and it is possible that a small initial R wave would have shown on a longer run.

The lesions produced by isoproterenol were much smaller than those produced by the diet, so it is perhaps not surprising that the injected animals produced less pronounced T wave inversion. The injections caused a striking tachycardia and it has been found the drug also lowers blood pressure while increasing the oxygen needs of heart muscle by direct action (Rona *et al.* 1959). It is likely that myocardial ischaemia is the result, repeated injections leading to patchy sub-

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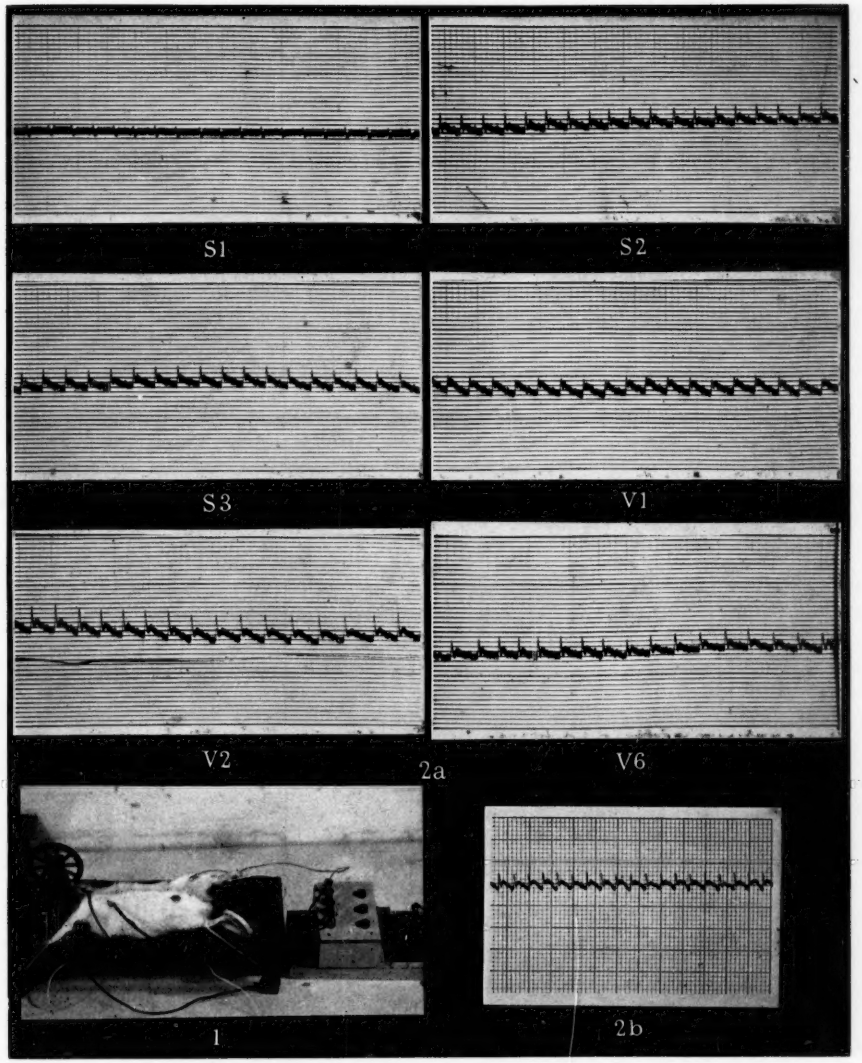
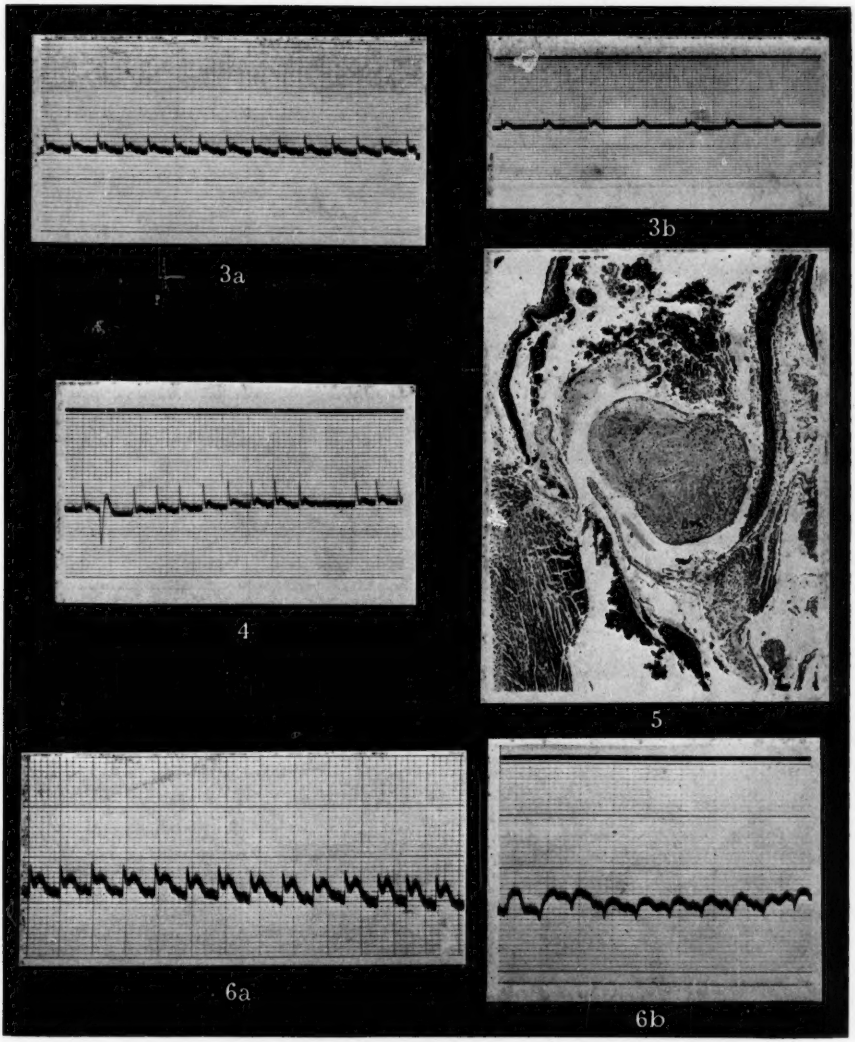
EXPLANATION OF PLATE.

FIG. 1.—A rat secured in the clamp with the electrodes inserted.
FIG. 2*a*.—ECGs of a normal rat using the photographic method. Leads S1, 2 and 3, V1, 2, 3, 4 and 6.
FIG. 2*b*.—ECG of a normal rat using the direct writing method.
FIG. 3*a*.—ECG of normal rat before pressure on neck. Lead V2.
FIG. 3*b*.—ECG of same rat after pressure on neck showing bradycardia. Lead V2.
FIG. 4.—ECG of normal rat after pressure on neck showing an ectopic beat, a dropped beat and a prolonged PR interval. Lead V2.
FIG. 5.—Ball thrombus in the aorta of a rat fed "infarct producing" diet.
FIG. 6*a*.—ECG showing normal pattern. Lead V1.
FIG. 6*b*.—ECG of same rat with infarct showing prominent Q waves. Lead V1.
FIG. 7*a*.—ECG showing normal pattern. Lead V2.
FIG. 7*b*.—ECG of same rat with infarct showing inversion of T wave. Lead V2.
FIG. 8*a*.—ECG showing normal pattern. Lead V2.
FIG. 8*b*.—ECG of same rat with infarct showing giant inverted T waves. Lead V2.
FIG. 9*a*.—ECG showing normal pattern. Lead V2.
FIG. 9*b*.—ECG of same rat with infarct showing terminal inversion of T wave. Lead V2.
FIG. 10.—ECG of rat with infarct showing two premature ventricular contractions and inverted T waves. Lead V2.


S1
S2
S3
V1
V2
V6
2a
1
2b



3a

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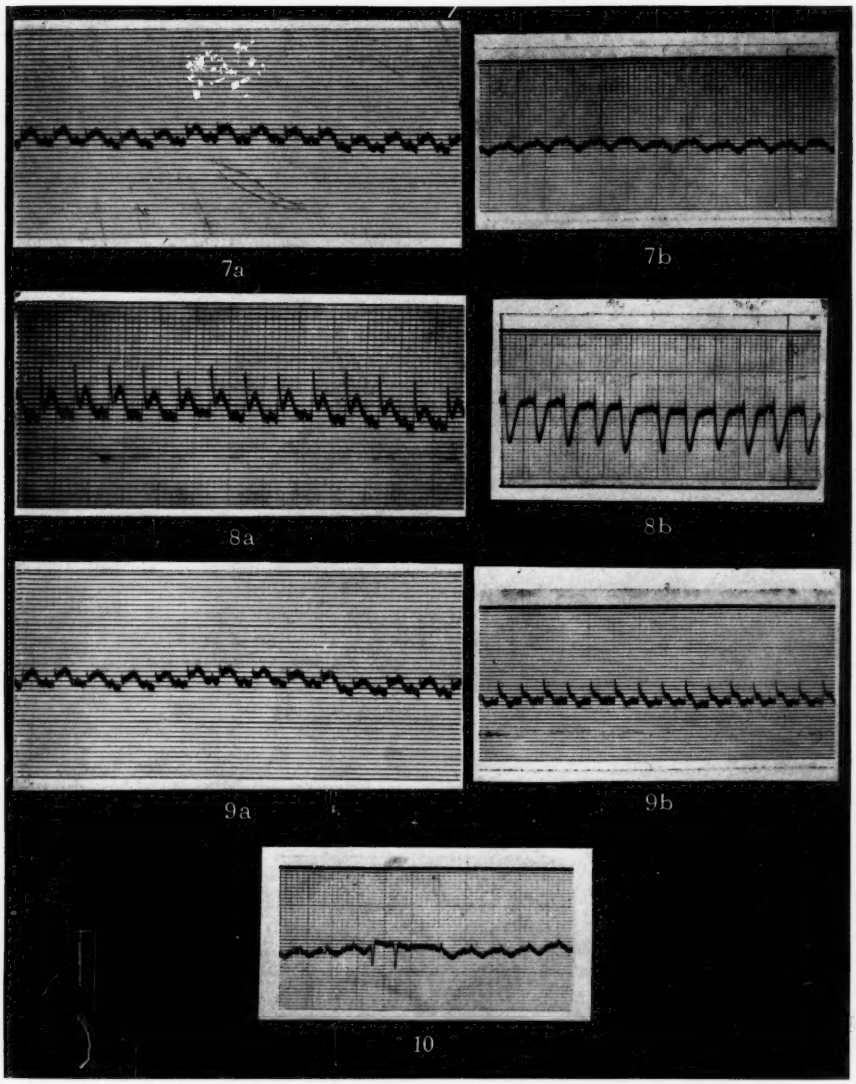
4

5

6a

6b



7a

7b

8a

8b

9a

9b

10

Hill, Howard and Gresham.

endocardial infarction. Our ECG findings in a small number of animals treated in this way are in keeping with such a course of events.

SUMMARY

Electrocardiograms were taken of rats which were normal or subjected to treatments which produce myocardial infarction, either by feeding a diet containing 40 per cent butter, 5 per cent cholesterol, 2 per cent cholic acid and 0·3 per cent thiouracil, or by injecting isoproterenol.

In normal rats, elevation of the S–T segment was a constant feature. No evidence was obtained for the existence of the N wave claimed by Heise and Kimbel (1955).

Using the dietary method, 5 out of 25 rats had myocardial infarction. All, except one with recent infarction, had abnormal electro-cardiograms consisting chiefly of T wave inversion which appeared from 15–40 days before death.

Rats injected with isoproterenol had small foci of sub-endocardial necrosis and showed electrocardiograms with inverted T waves.

It was concluded that electrocardiography can provide early evidence of infarction in the living rat.

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THE PROTECTION OF TISSUE CULTURES BY ISATIN β-THIOSEMICARBAZONE FROM THE CYTOPATHIC EFFECTS OF CERTAIN POX VIRUSES

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THE ability of isatin β-thiosemicarbazone (Fig. 1, 1) to protect mice from the lethal effects of inoculation with various pox viruses provides one of the few known examples of a compound which exhibits potent antiviral activity *in vivo*. The implication that this substance might be of clinical utility has encouraged extensive investigation in animals of the antiviral effects produced both by this and by many related compounds. Such studies have defined the conditions under which isatin β-thiosemicarbazone must be administered to achieve effective treatment of experimental pox virus infections, and have also served to emphasize its unique antiviral properties (Thompson, Minton, Officer and Hitchings, 1953 ; Bauer, 1955 ; Bauer and Sheffield, 1959 ; Campaigne, Thompson and van Werth, 1959 ; Bauer and Sadler, 1960). It is surprising, therefore, that reports of the antiviral effects of isatin β-thiosemicarbazone in tissue culture are entirely lacking. Not only would *in vitro* methods enable this compound to be tested for activity against viruses for which no suitable laboratory animal is available, but it is reasonable to suppose that any antiviral effects observed with pox viruses would lead to improved criteria for the recognition *in vitro* of compounds likely to possess antiviral activity *in vivo*. Furthermore, the advantages of a homogeneous host cell population admitting of easy manipulation and enumeration offers opportunities for the study of the mode of action of isatin β-thiosemicarbazone, at both the biological and biochemical levels, far superior to those available to earlier workers. Considerations such as these have prompted the experiments described in this paper. The effect of isatin β-thiosemicarbazone on the cytopathogenicity of 3 pox viruses for rabbit embryo kidney cells has been studied and the manner in which the compound provides protection for the cells has been investigated. In addition the protective effects of 3 closely related compounds, isatin β-semicarbazone (Fig. 1, 2), isatin β-amidinohydrazone (Fig. 1, 3) and the sodium salt of 7-carboxyisatin β-thiosemicarbazone (Fig. 1, 4) have been examined.

MATERIALS AND METHODS

Viruses

The IHD strain of mouse-adapted neurovaccinia virus and the Sandom strain of ectromelia virus were used. Both strains have been maintained by serial mouse brain passage in this laboratory for several years. The Utrecht strain of rabbitpox virus was supplied as rabbit testis homogenate by Dr. E. A. Boulter of the Microbiological Research Establishment, Porton.

Tissue cultures

The ERK I line of cells derived from embryo rabbit kidney (Westwood, McPherson and Titmuss, 1957) was used in all experiments. The cells were propagated by a method previously described (Churcher, Sheffield and Smith, 1959) with the single exception that papain digest broth was substituted for the peptic digest used heretofore. The Earle's solution (less bicarbonate), lactalbumin hydrolysate and papain digest broth were mixed, sterilized by heat and designated ELP.

Plaque counting method

A modification of the method described by Postlethwaite (1959) was used. Confluent cultures of ERK I cells in 8 oz. Pyrex babies' feeding bottles were inoculated with 0·5 ml. of

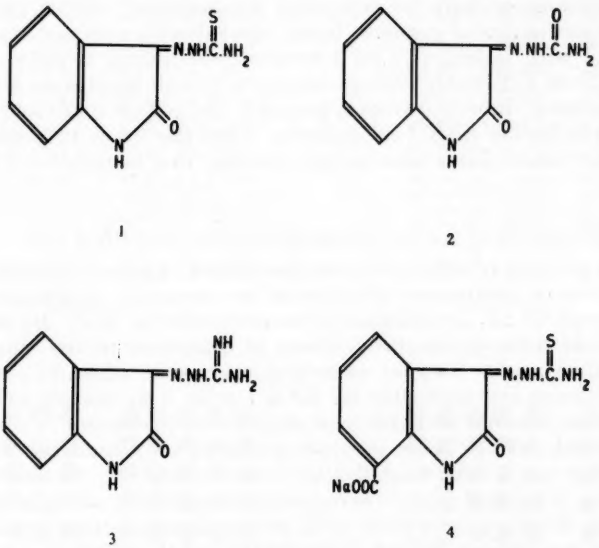


FIG. 1.—Isatin β-thiosemicarbazone and related compounds.

virus suspension appropriately diluted in growth medium. Four hr. at room temperature were allowed for absorption of virus ; 10 ml. of growth medium were then added to each culture and the bottles were returned to the incubator. After 40 hr. incubation the medium was decanted and replaced with 3 ml. of Ziehl-Neelsen carbol-fuchsin diluted 1 : 9 with 0·23 M sodium chloride solution. The stain was removed after 10 min. and the plaques were counted.

Intracerebral titrations in mice

The infectivity of ectromelia fluids was determined by intracerebral inoculation of mice and evaluation of the D_0 (Bauer, 1960).

Staining technique

Tube cultures were fixed with 95 per cent ethanol, dehydrated and treated with a 50 : 50 ethanol-ether mixture. Each tube was then filled with collodion solution. After 2 hr. the collodion was decanted, leaving a continuous film around the tube wall. To facilitate recognition of the cell-bearing area the film was stained with haematoxylin *in situ*. It was then detached from the tube and the stained area was spread on a glass slide. After counterstaining

with eosin the preparation was dehydrated as far as 95 per cent ethanol, treated for 10 min. with *n*-butanol, cleared in xylene and mounted in canada balsam.

EXPERIMENTAL

The growth of pox viruses in ERK I cell cultures

Serial 10-fold dilutions of brain homogenate from a mouse infected intracerebrally with neurovaccinia virus were made in growth medium. Two ml. of each dilution were added to each of 5 ERK I cell cultures containing approximately 10^5 cells per culture. The cultures were incubated at 37° with continuous rotation and examined daily for cytopathic degeneration. Similar titrations were done with homogenates of a mouse brain infected with ectromelia and a rabbit testis infected with rabbitpox. All 3 viruses grew readily in ERK I cells with cytopathic effects sufficiently pathognomonic to permit immediate differentiation of the virus strains. Infective virus appeared in the culture fluids and was serially transmissible in further ERK I cell cultures. After the 3rd or 4th serial passage of each virus the culture fluids were pooled severally and stored at −20° for use as seed virus.

Titration of the toxicity of isatin β-thiosemicarbazone for ERK I cells

A 200 μM solution of isatin β-thiosemicarbazone in growth medium was made by the addition of appropriate amounts of bovine serum and bicarbonate to a previously prepared 227 μM solution of the compound in ELP. By dilution with growth medium serial arithmetic dilutions of compound in the range of 200 to 10 μM were then made. Two ml. of each dilution were added to each of 2 tube cultures containing approximately 10^5 ERK I cells. The cultures were incubated at 37° on a roller machine and examined daily for toxic changes. That molarity of compound which after 7 days incubation caused only minimal morphological changes visible at 54-fold magnification was termed the maximum tolerated concentration. A value of 40 μM was regularly obtained for isatin β-thiosemicarbazone. Similar titrations were done with each compound examined for antiviral activity. The maximum tolerated concentrations of the 4 substances used in this investigation are recorded in Table I.

The protective effect of isatin β-thiosemicarbazone in cultures infected with pox viruses

The ability of isatin β-thiosemicarbazone to protect ERK I cells from the cytopathic effects of pox viruses was studied in chessboard-type experiments. ERK I tube cultures each containing approximately 10^5 cells were arranged in 10 columns of 15 tubes in a roller drum. The medium was then removed from each culture. To the first 5 columns 5 solutions of isatin β-thiosemicarbazone in concentrations of 80, 24, 8, 2·4 and 0·8 μM in growth medium were allocated. One ml. of each solution was pipetted into each tube of the corresponding column. To every tube of 5 further columns was added 1 ml. of growth medium without the compound. The cultures were then incubated at 37° on a roller machine for 30 min. During this time 14 serial 3·16-fold dilutions of virus were prepared in growth medium. One dilution was allocated to each of the first 14 rows and distributed in 1 ml. volumes to each tube therein. One ml. of growth medium without virus was added to each tube in the last row. The " chessboard " thus prepared contained

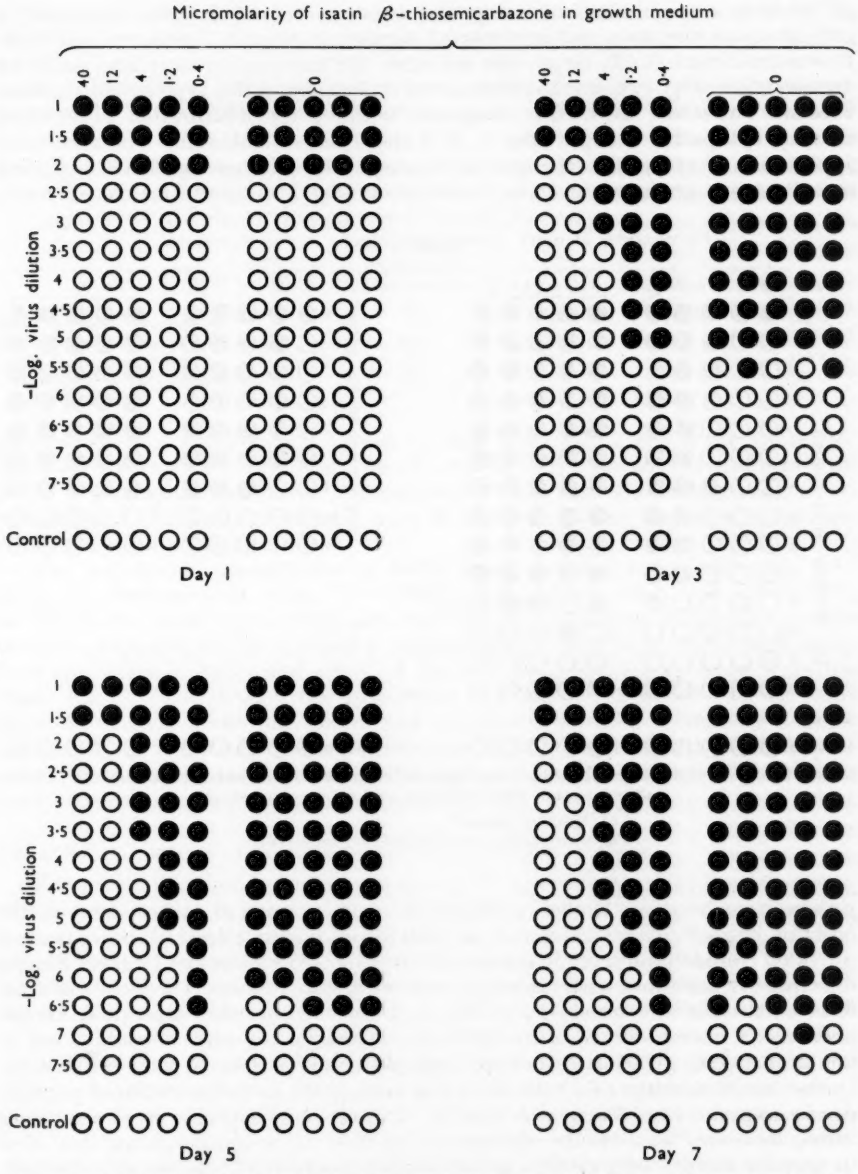


FIG. 2.—The distribution of healthy and degenerated cultures in a chessboard experiment 1, 3, 5 and 7 days after infection with neurovaccinia virus.

○ Healthy culture.
● Culture showing cytopathic degeneration.

all possible combinations of compound concentration and virus inoculum, a parallel virus titration and controls of compound toxicity, medium and cells. It was incubated at 37° on a roller machine, the tubes being examined daily for degeneration. Fig. 2 shows the results of an experiment of this type in which neurovaccinia virus was used. The diagrams indicate the distribution of cultures showing cytopathic changes after 1, 3, 5 and 7 days incubation. The successive appearance of cytopathic changes in titration tubes containing decreasing virus inocula was reproduced in those tubes containing low concentrations of isatin

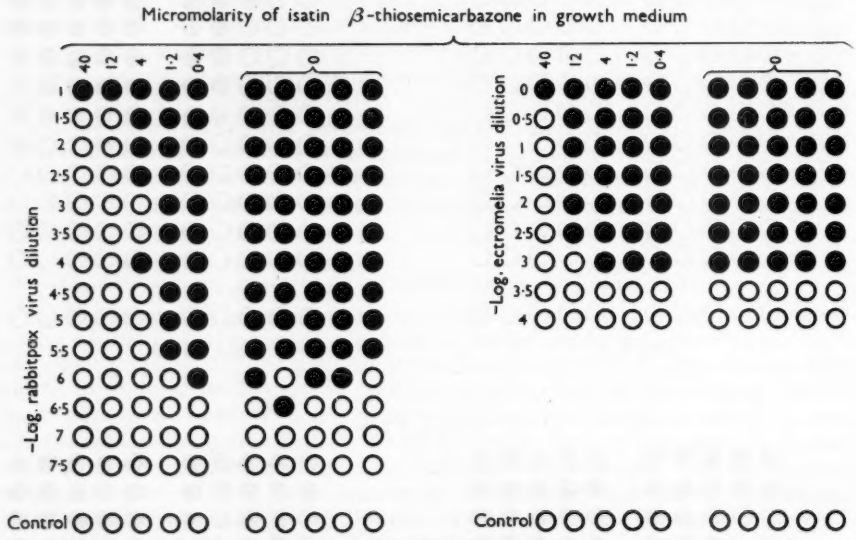


FIG. 3.—The distribution of healthy and degenerated cultures in chessboard experiments 7 days after infection with rabbitpox and ectromelia viruses.

○ Healthy culture.
● Culture showing cytopathic degeneration.

β-thiosemicarbazone. Higher concentrations of compound either delayed the onset of cytopathic degeneration or, as with the maximum tolerated concentration, provided complete protection against all virus inocula except the largest. Similar experiments were done with rabbitpox and ectromelia viruses. The results of these tests on the 7th day are shown in Fig. 3. It can be seen that essentially similar phenomena were observed, although with ectromelia the virus inoculum was of low titre and the protective effects of isatin β-thiosemicarbazone were less striking. In the case of rabbitpox such an effect was anticipated from the results of previous *in vivo* experiments (Bauer *et al.*, 1959). Protection of cultures from ectromelia virus, however, was wholly unexpected as tests of activity against this virus in mice have repeatedly given negative results (Beck, 1957 ; Bauer, unpublished).

An estimate of the protection afforded to tissue cultures by various concentrations of antiviral agent may be made from the results of these experiments. By subtracting the lowest dilution of virus the cytopathic effects of which are com-

pletely inhibited from the titre of the undiluted inoculum the number of TCD_{50} of virus from which the compound provides protection may be obtained. The results of such calculations made from 7th day readings of experiments with isatin β-thiosemicarbazone and neurovaccinia, rabbitpox and ectromelia viruses are given in the Table. These values are those obtained with the maximum tolerated

TABLE.—*The Maximum Concentrations of Isatin β-thiosemicarbazone and Related Compounds Tolerated by ERK I Cells and the Largest Inocula of Pox Viruses Against Which Such Concentrations Provide Protection*

	Maximum tolerated concentration	Neurovaccinia	Rabbitpox	Ectromelia
	μM	TCD_{50}	TCD_{50}	TCD_{50}
Isatin β-thiosemicarbazone	40	$10^{4\cdot7}$	$10^{4\cdot6}$	$10^{2\cdot8}$
Isatin β-semicarbazone	200	<10	<10	10
Isatin β-amidinohydrazone	800	<10	<10	$10^{1\cdot2}$
7-carboxyisatin β-thiosemicarbazone, sodium salt	800	<10	<10	<10

concentration of compound. As can be seen from Fig. 2 and 3 the values for lower concentrations are smaller. Also in the Table are the results obtained with isatin β-semicarbazone, isatin β-amidinohydrazone and the sodium salt of 7-carboxyisatin β-thiosemicarbazone, none of which was found to possess more than trivial antiviral properties.

The histological appearances of ERK I cell cultures inoculated with $10^{3\cdot7}$ TCD_{50} of neurovaccinia virus, $10^{3\cdot2}$ TCD_{50} of rabbitpox virus and $10^{2\cdot6}$ TCD_{50} of ectromelia virus respectively and maintained for 3 days in normal growth medium are shown in Fig. 4, 6 and 8. Cytopathic changes have almost completely destroyed these cultures and many of the cells have become detached from the glass. Fig. 5, 7 and 9 show the appearance of cultures inoculated with the same amounts of each virus but maintained in growth medium containing a 40μM concentration of isatin β-thiosemicarbazone. In these cultures the cells appear as confluent healthy monolayers and the cytopathic effects of each of the viruses have been completely inhibited by the compound.

The action of isatin β-thiosemicarbazone on pox viruses in vitro

The possibility that both the *in vivo* and *in vitro* effects of isatin β-thiosemicarbazone were due to extracellular destruction of virus infectivity necessitated a comparison of the rate of virus inactivation in growth medium with that in growth medium containing the compound in the highest concentration used. In the first inactivation experiment a neurovaccinia suspension containing approximately 10^3 plaque-forming units per ml. was made in growth medium. Four ml. of this suspension were pipetted into each of 2 universal containers. To one was added 4 ml. of an 80 μM solution of isatin β-thiosemicarbazone in growth medium, giving a final concentration of 40 μM, and to the other was added 4 ml. of normal growth medium. The contents of the tubes were mixed and held in a water bath at 37°. One ml. samples were removed from both containers after various time intervals, diluted with 4 ml. of growth medium and used to initiate plaques in ERK I cell monolayers. The logarithm of the number of plaques produced by each sample had a linear regression on time (Fig. 10). The regression coefficients in the absence (−0·0989) and in the presence (−0·1048) of isatin β-thiosemi-

carbazone were significantly different from zero ($P < 0{\cdot}001$), showing that thermal inactivation was taking place, but did not differ significantly from each other ($P = 0{\cdot}7$). The virus was therefore not being inactivated by the compound.

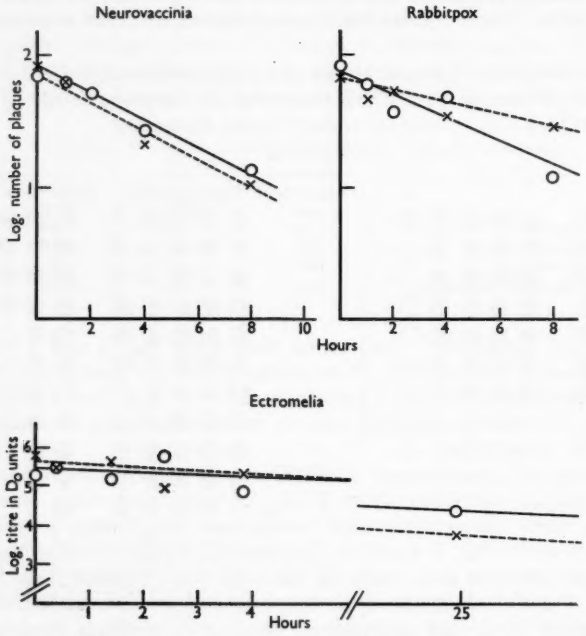


FIG. 10.—The rates of inactivation of neurovaccinia, rabbitpox and extromelia viruses when incubated at 37° in growth medium and in growth medium containing a 40 μM concentration of isatin β-thiosemicarbazone.

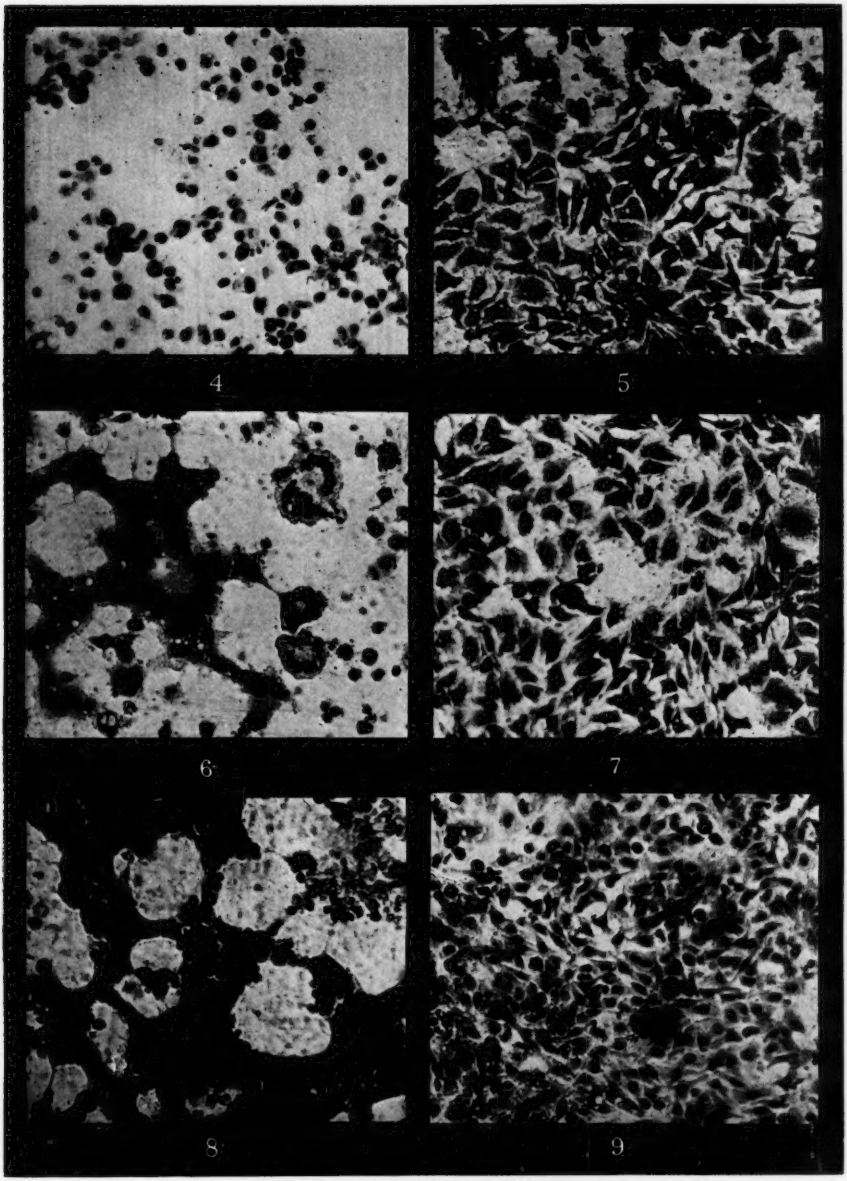
○———○ Virus suspensions in growth medium.
×– – –× Virus suspensions in growth medium containing isatin β-thiosemicarbazone.

Similar results were obtained with rabbitpox virus (Fig. 10). The regression coefficients were highly significant ($P < 0{\cdot}001$), indicating the occurrence of thermal inactivation, and the value obtained in the presence of the compound ($-0{\cdot}0790$) was less negative than in the control series ($-0{\cdot}1189$), showing that the compound could not be causing any inactivation.

EXPLANATION OF PLATE

The photomicrographs show the appearances of ERK 1 cell monolayers 3 days after infection with pox viruses. Advanced cytopathic changes are evident in cultures maintained in normal growth medium. Those cultures maintained in growth medium containing a 40 μM concentration of isatin β-thiosemicarbazone are unaffected. Haematoxylin and eosin : ×150.

FIG. 4.—Neurovaccinia virus inoculum : $10^{3{\cdot}7}$ TCD_{50}. Normal growth medium.
FIG. 5.—As Fig. 4 but maintained in medium containing isatin β-thiosemicarbazone.
FIG. 6.—Rabbitpox virus inoculum : $10^{3{\cdot}2}$ TCD_{50}. Normal growth medium.
FIG. 7.—As Fig. 6 but maintained in medium containing isatin β-thisosemicarbazone.
FIG. 8.—Ectromelia virus inoculum : $10^{2{\cdot}6}$ TCD_{50}. Normal growth medium.
FIG. 9.—As Fig. 8 but maintained in medium containing isatin β-thiosemicarbazone.



Sheffield, Bauer and Stephenson.

The slower and less regular growth of ectromelia in ERK I cells has precluded the use of plaque counting methods with this virus. Samples obtained from incubated suspensions of virus in growth medium and in growth medium containing a 40 μM concentration of isatin β-thiosemicarbazone were therefore titrated for infectivity by intracerebral inoculation of mice. In order to avoid repeated determinations of the LD_{50} by the inoculation of serial decimal dilutions the inactivation curves were obtained from the single-dilution D_0 titres by the method of regression coefficient transformation (Bauer, 1960). The results are shown in Fig. 10. The regressions of the titres on time are linear ; the regression coefficients (−0·0012 and −0·0022) are highly significant ($P < 0{\cdot}001$, 0·05), but do not differ significantly from each other ($P = 0{\cdot}3 - 0{\cdot}2$). There is therefore no evidence that isatin β-thiosemicarbazone can inactivate ectromelia virus *in vitro*.

The effect of isatin β-thiosemicarbazone on the adsorption of pox viruses to ERK I cells

An alternative explanation of the protective effect of isatin β-thiosemicarbazone was the possibility that the compound inhibited the adsorption of virus to susceptible tissue culture cells. Experiments were therefore done to determine whether the rate of adsorption of pox viruses to ERK I cell monolayers was retarded by the presence of the compound in the inoculum. A suspension of neurovaccinia virus containing approximately 400 plaque-forming units per ml. was prepared in growth medium. Five ml. of this suspension were pipetted into each of 2 universal containers and to one was added 5 ml. of growth medium, and to the other 5 ml. of an 80 μM solution of isatin β-thiosemicarbazone in growth medium. Each suspension was then used to inoculate a batch of 12 ERK I cell cultures with 0·5 ml. per culture. The cultures were incubated at 37°, and after ½, 1, 2 and 4 hr. 3 cultures were removed from each batch and washed 5 times with 20 ml. of ELP. Ten ml. of growth medium were then added to each washed culture and incubation was resumed. After 40 hr. incubation all the cultures were stained and examined for plaques. The results of 2 experiments of this type, one with neurovaccinia and one with rabbitpox, are given in Fig. 11. The average numbers of plaques produced with each adsorption period are plotted against adsorption time. It is apparent that the rate of adsorption of neither virus is affected by the presence of the maximum tolerated concentration of the compound. Attempts to perform this type of experiment with ectromelia virus have so far been unsuccessful.

DISCUSSION

Investigation of the properties of isatin β-thiosemicarbazone in tissue cultures inoculated with neurovaccinia, rabbitpox and ectromelia viruses has shown that this compound affords protection from the cytopathic effects of each of these infectious agents. The very substantial protection observed with neurovaccinia and rabbitpox viruses is correlated with the ability of the compound to provide protection from the effects of these viruses *in vivo*. In the case of ectromelia, however, only moderate protection of tissue cultures is apparent and this is associated with absence of antiviral effects *in vivo*. It seems reasonable to suppose that tissue culture provides a milieu in which the susceptibility of viruses to antiviral agents is greater than that which obtains in the intact host. Thus only

effects comparable in extent with those observed with neurovaccinia and rabbitpox viruses are likely to be indicative of antiviral effects *in vivo*. A consideration of these features assumes particular importance in tissue culture screening programmes designed to detect compounds effective against those viruses for which no small laboratory host is available. Compounds showing only low antiviral activity in tissue culture may reasonably be excluded from testing in large animals, with consequent economy.

Investigations of the mode of action of isatin β-thiosemicarbazone have been greatly facilitated by the use of modern tissue culture methods. By means of plaque counting techniques it has been possible to show that the antiviral activity

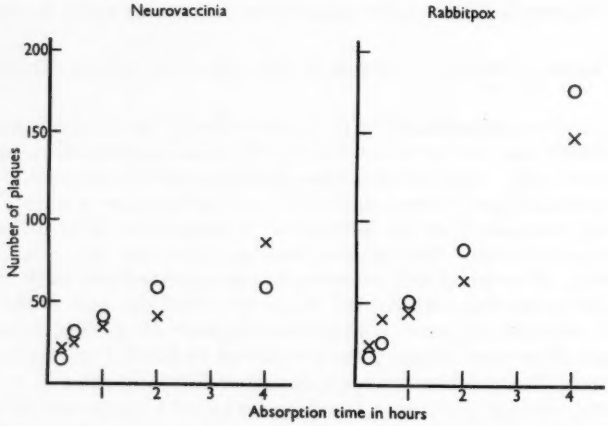


FIG. 11.—The adsorption of neurovaccinia and rabbitpox viruses on to ERK1 cell monolayers from virus suspensions in growth medium and in growth medium containing a 40 μM concentration of isatin β-thiosemicarbazone.

○ Virus suspensions in growth medium.
× Virus suspensions in growth medium containing isatin β-thiosemicarbazone.

of the compound is due neither to destruction of virus infectivity *in vitro* nor to inhibition of virus absorption to susceptible cells. The inference must therefore be drawn that the protection of cultures by isatin β-thiosemicarbazone results from some process taking place within the cells. This view is supported by current experiments which indicate almost complete inhibition of virus replication in infected cells maintained in media containing the maximum tolerated concentration of compound. Whether this be due to an effect on the virus particle itself or to interference with metabolic processes used in the production of new virus is, as yet, unknown.

The failure of isatin β-semicarbazone and isatin β-amidinohydrazone to protect cultures from the cytopathic effects of any of the 3 pox viruses tested emphasizes the singular properties of the thio compound. In the case of the 7-carboxy derivative major changes in biological properties may reasonably be expected to result from the size of the substituent and the presence of an electrical charge, but with the semicarbazone and the amidinohydrazone only the sulphur atom has been

replaced. The loss of antiviral activity consequent upon such small changes of structure and the inability of isatin β-thiosemicarbazone to inhibit the replication of almost all other viruses against which it has been tested suggest that its action in the host cell is a highly specific one. An investigation of its site and mode of action within cells infected with pox viruses may prove to be a most interesting aspect of antivirology.

We are indebted to Dr. P. W. Sadler of the Courtauld Institute of Biochemistry for the sample of 7-carboxyisatin β-thiosemicarbazone used in these experiments.

SUMMARY

Subtoxic amounts of isatin β-thiosemicarbazone incorporated into the growth medium of ERK I cell cultures prevented the appearance of cytopathic changes after infection with neurovaccinia, rabbitpox and ectromelia viruses. The compound did not destroy virus infectivity *in vitro* nor did it prevent adsorption of virus to tissue culture cells. The conclusion is reached that the protective effect of isatin β-thiosemicarbazone is due to intracellular inhibition of virus replication.

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ACTION OF PHOSPHOLIPIDS ON THE CYTOTOXIC EFFECT OF RABBIT ANTIBODIES AGAINST EHRLICH ASCITES TUMOUR CELLS*

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STUDIES on the cytotoxic action of antibodies, plasmin and streptococcal haemolysins on Ehrlich ascites tumour cells (E-cells) have been recently described (Ginsburg, 1959; Ginsburg and Grossowicz, 1960; Ginsburg and Ram, 1960). It was found that the cytopathogenic changes induced in the tumour cells by streptococcal haemolysins were morphologically very similar to those caused by anti-Ehrlich ascites tumour rabbit serum (AE), the action of the latter is known to be complement dependent (Flax, 1956; Goldberg and Green, 1959).

It is known that both the haemolytic as well as the cytopathogenic effect caused by streptococcal haemolysins can be inhibited by antihaemolytic agents such as cholesterol, lecithin, congo red and trypan blue (Van Heyningen, 1950; Bernheimer, 1954; Ginsburg, 1959; Ginsburg and Grossowicz, 1960). On the other hand, the cytotoxic and haemolytic effect of antibodies can be inhibited by agents known to destroy or inhibit complement action, chelating agents, plasmin, heparin etc. (Gorrill and Hobson, 1952; Pillemer, Ratnoff, Blum and Lepow, 1953; Mayer, 1958; Ginsburg and Ram, 1960).

The present study shows that the cytotoxic action of antibodies produced in the rabbit against Ehrlich ascites tumour cells can be inhibited by various phospholipids and some of their constituents as well as by agents known to inhibit streptococcal haemolysins. In addition the inhibition of immune haemolysis by phospholipids will be described.

MATERIALS AND METHODS

Tumour cells

Ehrlich ascites tumour cells (Landchütz strain) were maintained by weekly passages in white mice. The cells (7 days old) were washed with buffered saline (24 g. $Na_2HPO_4.12H_2O$, 9 g. KH_2PO_4 and 4·5 g. NaCl per litre) pH 7·4 or in 0·15 M saline pH 7·4 and were kept in ice until used. The cell suspensions used (10^7/ml.) usually contained 2–4 per cent dead cells as determined by staining with trypan blue (1 per cent).

Antisera

Anti-tumour antibodies were produced in rabbits according to the method of Salk and Laurent (1952). Three to 4 weeks following the first injection the sera of the rabbits revealed cytotoxic action on E-cells up to a titre of 1 : 20 which was found to be complement (C′) dependent. The sera were stored at −20°.

* Supported in part by The Howard Netzky Memorial Club, Chicago, Illinois, Florence Albin Cancer Club, Chicago, Illinois and Mr. Robert Gould of Bridgeport, Connecticut.

Materials tested

Lecithin crude (animal, vegetable or egg) was obtained from N.B.C. Cleveland, Ohio, U.S.A.

Lecithin purified (Phosphatidyl choline) was obtained from egg yolk according to the method of Hanahan, Turner and Jayco (1951).

Lysolecithin was obtained by treating crude lecithin with phosphorylase A obtained from pancreas according to the method of Belfanti, Ercoli and Francioli (1945).

Phosphatidic acid was obtained by treating crude lecithin with phosphorylase C according to the method of Kates (1956).

Cephalins.—Phosphatidyl serine, phosphatidyl ethanolamine and phosphatidyl inositol were obtained from calf brain according to the method of Folsch (1942).

All the above materials were prepared by Dr. A. Rimon of the Marcus Memorial Blood Institute, Jaffa, Israel.

Cerebroside was isolated from spinal cord and purified on magnesium silicate columns. This material was prepared by Dr. M. Statter of the Department of Physiological Chemistry, Hebrew University Hadassah Medical School, Jerusalem.

Proteolipid was obtained from human brain by the method of Folsch (1942). This material was prepared by Dr. C. Tal of the Department of Experimental Neurology, Hebrew University Hadassah Medical School.

Cholesterol was prepared by dissolving the material in methanol. The solution was then poured into boiling water.

Phospho-DL-serine and *poly phospho-DL-serine* were synthesized by Dr. Y. Lapidot of the Department of Biological Chemistry of the Hebrew University.

All materials were dissolved in buffered saline or in saline.

Inhibition studies

The materials were incubated for 10 min. at 37° with AE prior to the addition of tumour cells. In most of the experiments a 10 per cent final concentration of AE was employed and the volume of the system was made up to 1·0 ml. with saline. This amount of AE was sufficient to kill 100 per cent of tumour cells after 30 min. incubation at 37°. To test whether or not the inhibition of AE could be reversed by complement or cations, fresh guinea pig serum or $1{\cdot}3 \times 10^{-3}$ M Ca^{++} plus $1{\cdot}3 \times 10^{-3}$ M Mg^{++} were added to the inhibited system. Results were read 30 min. after the addition of cells, complement or cations. In each experiment 500 cells were counted and the number of dead cells expressed as percent inhibition was calculated.

RESULTS

Inhibition of AE by anti-haemolytic agents

Table I shows that marked inhibition of AE action was obtained by congo red, trypan blue and crude lecithin. Inhibition by cholesterol was transitory lasting 20–25 min. after which the majority of tumour cells died. No inhibition occurred if the various materials were preincubated with E-cells prior to the addition of AE. Whenever inhibition occurred, the E-cells appeared in large clumps showing that agglutination of the cells was unaffected. Inhibition by all materials was completely reversed by guinea pig serum. These results suggested that the various materials inhibited complement (C′) essential for the cytotoxic action of AE. Experiments with crude lecithin showed that while at a concentration of approximately 0·00005M the compound inhibited AE action, higher concentrations accelerated the death of the tumour cells. These results suggested that the crude lecithin employed contained materials toxic for E-cells.

Attempts were made to identify the fractions in crude lecithin responsible for inhibiting AE action. Using paper chromatographic techniques for the separation of lipids (Marinetti, Erbland and Kochen, 1957) it was found that crude lecithin contained at least 6 fractions, 2 of which were identified as lecithin and lysolecithin. No attempts were made to identify the other fractions present.

TABLE I.—*Inhibition of AE Action by Anti-haemolytic Agents*

Reaction mixture	Percent inhibition
Tumour cells + AE*	0
+	
Cholesterol $3 \cdot 3 \times 10^{-3}$ M	80
+ Complement†	0
Congo red $6 \cdot 7 \times 10^{-4}$ M	100
+ Complement	0
Trypan blue 10^{-3} M	100
+ Complement	0
Lecithin crude 5×10^{-5} M	80
$6 \cdot 7 \times 10^{-5}$ M‡	0
Lecithin crude 5×10^{-5} M + Complement	0

* AE 10 per cent in saline was incubated for 10 min. at 37° with the various materials before the addition of E-cells (10^7/ml.). This amount of AE killed 100 per cent of the tumour cells within 30 min. of incubation at 37°. The results were read 30 min. after the addition of the cells. The figures represent an average of 5 experiments; similar results were obtained with phosphate buffer as diluent, the amount of AE needed to cause 100 per cent mortality was, however, much higher (see Fig. 2).

† 10 per cent fresh guinea-pig serum.

‡ These concentrations of lecithin markedly accelerated the death of tumour cells (see text).

The experiments described in Table I were repeated with purified lecithin, lysolecithin and phosphatidic acid.

Table II shows that both lysolecithin and phosphatidic acid inhibited AE action while purified lecithin was not effective even at high concentrations

TABLE II.—*Inhibition of AE Action by Phospholipids*

Reaction mixture	Percent inhibition
Tumour cells + AE*	0
+	
Lecithin purified 10^{-2} M	0
Lysolecithin 2×10^{-4} M	85
$2 \cdot 2 \times 10^{-4}$ M	0†
Phosphatidic acid $6 \cdot 7 \times 10^{-4}$ M	70
10^{-3} M	0†
Tween 40 500 μg./ml.‡	0
80 200 μg./ml.‡	0
Taurocholate 5×10^{-4} M	0
Cortisone 5×10^{-4} M	0
Progesterone 5×10^{-4} M	0
Testosterone 5×10^{-4} M	0

* As in Table I.

† These concentrations of materials accelerated the death of the tumour cells.

‡ Polyoxyalkylene sorbitan mono-oleate (Tween 80) and palmitate (Tween 40). The concentration of both materials was expressed in μg./ml. since the exact molecular weight is not known. The concentrations used were below the toxic level for E-cells.

(0·01M). These results suggest that inhibition of AE by crude lecithin was probably not due to lecithin. Experiments with lysolecithin and phosphatidic acid, both known to haemolyse red blood cells of various animal species, showed that inhibition by these materials could be obtained only by some critical concentrations, beyond which these materials exerted high toxicity on E cells when applied with subtoxic concentrations of AE, which suggested that the inhibition of AE was probably due to contamination with lysophosphatides.

Since both lysolecithin and phosphatidic acid are surface active agents, the possibility existed that the inhibition of AE caused by these compounds was due to competition with complement for some substrates located at the cell membrane. It was however found that subtoxic concentrations of Tween 40, Tween 80 and taurocholate as well as of cortisone, progesterone and testosterone (Table II) also known to haemolyse RBC or injure E-cells (Leonard and Rotstein, 1953) failed to inhibit AE action.

Inhibition of AE by cephalins

Coburn, Graham and Haninger (1954), Jaques (1958), Ganley, Grassle and Robinson (1958) and Ganley, Robinson and Blodzinski (1959) have described the anti-anaphylactic and anti-allergic action of fractions obtained from egg yolk, peanut oil, soy lecithin etc. Since egg yolk is known to be rich in phospholipids, various cephalins, some of their constituents (ethanolamine, phosphoserine, inositol) and several phosphoric acid esters, cerebroside and proteolipid were tested for their capacity to inhibit AE action. Table III shows that AE action was

TABLE III.—*Inhibition of AE by Cephalins and Cerebrosides*

Reaction mixture	Percent inhibition
Tumour cells + AE*	0
+	
Phosphatidyl serine 4×10^{-4} M	100
Phosphatidyl ethanolamine 2×10^{-4} M	100
Phosphatidyl inositol 5×10^{-4} M	100
Cerebroside 4×10^{-5} M	100
Proteolipid 150 μg./ml.	100
Phospho DL-serine 10^{-2} M	100
DL-serine 2×10^{-2} M	0
Poly-phospho DL-serine 2×10^{-3} M	100
Ethanolamine 2×10^{-2} M	85
Inositol 4×10^{-2} M	0
Dimethyl ethanolamine $6{\cdot}7 \times 10^{-2}$ M	0
Choline $6{\cdot}7 \times 10^{-2}$ M	0
α-glycerophosphate $6{\cdot}7 \times 10^{-2}$ M	0
Glucose 1-phosphate $6{\cdot}7 \times 10^{-2}$ M	0

* As in Table I.

completely blocked by the various cephalins, cerebroside, proteolipid, phosphoserine, polyphosphoserine and by ethanol amine. On the other hand, no inhibition was obtained by inositol, choline, dimethyl ethanolamine, glycerophosphate, glucose l-phosphate and DL-serine. The various inhibitors had no effect on agglutination of the cells and fresh guinea pig serum completely reversed the inhibition suggesting that the various inhibitors acted on complement.

Effect of Ca^{++} and Mg^{++} on inhibition of AE

The fixation of various complement components upon sensitized cells is known to be dependent on the presence of optimal concentrations of both Ca^{++} and Mg^{++} (Mayer, Osler, Biear and Heidelberger, 1946 ; Mayer, 1958). To test whether or not inhibition of AE action by the various inhibitors was due to their chelating properties, the action of $1{\cdot}3 \times 10^{-3}$ M Ca^{++} plus $1{\cdot}3 \times 10^{-3}$ M Mg^{++} was tested on the inhibited systems. This concentration of cations is the highest

not causing inhibition of AE. In addition, the action of these cations on the inhibition caused by Ethylene-diamine-tetra-acetic acid (EDTA) 10^{-3} M was also tried. The action of cations on some of the inhibitors is given in Table IV. It

TABLE IV.—*Effect of Mg^{++} and Ca^{++} on Inhibition of AE*

Reaction mixture	Percent inhibition
Tumour cells + AE*	0
+	
Cerebroside $2{\cdot}5 \times 10^{-4}$ M	100
+ Mg^{++}†	25
+ Ca^{++}†	100
+ Mg^{++} + Ca^{++}	22
+ inactivated rabbit serum (10 per cent)	100
Phosphatidyl serine 4×10^{-4} M‡	100
+ Mg^{++}	35
+ Ca^{++}	100
+ Mg^{++} + Ca^{++}	30
+ inactivated rabbit serum	35
EDTA 10^{-3} M	100
+ Mg^{++}	0
+ Ca^{++}	0
+ Mg^{++} + Ca^{++}	0
+ inactivated rabbit serum	0
Congo red $6{\cdot}7 \times 10^{-4}$ M§	100
+ Mg^{++}	100
+ Ca^{++}	100
+ Mg^{++} + Ca^{++}	100
+ inactivated rabbit serum	25

* Same as in Table I.

† $1{\cdot}3 \times 10^{-3}$ M of Ca^{++} or Mg^{++}.

‡ Similar results were obtained for phosphatidyl ethanolamine, phosphatidyl inositol, phosphoserine and poly phosphoserine.

§ Similar results were obtained for trypan blue and ethanolamine.

can be seen that except in the cases of Congo red, trypan blue and ethanolamine, inhibition by all other materials was reversed to a large extent by Mg^{++} alone. Ca^{++} had no effect on the various systems. On the other hand, either Ca^{++} or Mg^{++} completely reversed the inhibition caused by EDTA. Under the same experimental conditions Sr^{++}, Ba^{++}, Mn^{++}, Fe^{++} and Zn^{++} had no effect on the inhibition caused by either EDTA or the other inhibitors used. No reversion of the inhibition caused by cerebroside and proteolipid could be obtained by inactivated rabbit serum (final concentration 70 per cent). On the other hand, inhibition caused by EDTA, phosphatidyl serine, phosphatidyl inositol, congo red and trypan blue was completely reversed by serum. These results also suggested that the various inhibitors probably acted by binding Mg^{++} responsible for the action of complement. These findings are in agreement with the findings of Mayer *et al.*, (1946) and Mayer (1958) that the inhibition of complement by various inhibitors can be reversed by Mg^{++} but less effectively by Ca^{++}.

The Potentiating effect of Mg^{++} on AE action

During studies on the reversal of the inhibitory effect of phospholipids by Mg^{++} it was observed that the cytopathogenic changes induced in the cells as a result of the addition of Mg^{++} were more pronounced than those seen in cells exposed to AE alone. These findings led us to assume that Mg^{++} potentiated

the action of AE. To test this assumption, increasing concentrations of AE were incubated with E-cells in the presence of constant amount of Mg^{++} ($1{\cdot}3 \times 10^{-3}$ M). The experiments were conducted in saline, phosphate saline buffer or in the presence of various amounts of inactivated normal rabbit serum. Results are given in Fig. 1 and 2. It can be seen that Mg^{++} markedly increased the cytotoxic action of AE in all media used. It is of interest that 2–7 times the amount of AE is needed in order to obtain minimal cytotoxic action if the diluent used is either serum or phosphate buffer respectively as compared to experiments performed in saline. These results suggest that both serum and phosphate buffer are inhibitory to AE. Since the amounts of Mg^{++} sufficient to reverse the inhibition also potentiated the AE action, the possibility existed that the reversal of the

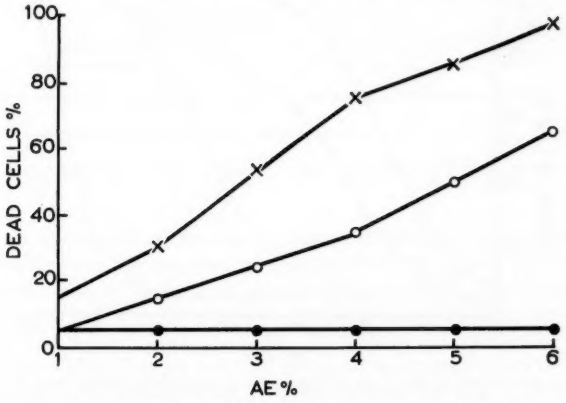

FIG. 1.—Effect of Mg^{++} on AE activity in saline.

●——● Inactivated rabbit serum diluted in saline + $1{\cdot}3 \times 10^{-3}$ M Mg^{++}.
○——○ AE diluted in saline.
×——× AE diluted in saline + Mg^{++}.

inhibition observed following the addition of Mg^{++} might be due to either the supply of Mg^{++} or to the potentiating effect of this cation. To test these assumptions increasing concentrations of cerebroside, phosphatidyl serine and phosphatidyl inositol were incubated for 5 min. at 37° with a constant amount of AE. After 5 min. E-cells were added followed 5 min. later by a constant amount of Mg^{++} ($0{\cdot}6 \times 10^{-3}$ M). It was found that approximately 50 per cent reversal of the inhibition exhibited by these materials still occurred even in the presence of excess inhibitors (2×10^{-3} M of cerebroside and 10^{-2} M of either phosphatidyl serine or phosphatidyl inositol), suggesting that no stoichiometric relation between Mg^{++} and inhibitor exists.

Inhibition of immune haemolysis by phospholipids

Studies on inhibition of complement action were usually performed on an immune haemolysis system (Mayer *et al.* 1946; Cushman, Becker and Wirtz, 1957). The action of the various phospholipids was therefore tried on this system. The system employed consisted of Amboceptor-Bacto anti-sheep haemolysin

(Difco) diluted 1 : 100 in saline, complement (guinea pig serum diluted 1 : 10) and sheep erythrocytes, a 20 per cent suspension. The various inhibitors, in 0·1 ml. volumes, were incubated for 5 min. at 37° with 0·1 ml. amboceptor and 0·1 ml. complement prior to the addition of 0·1 ml. red blood cells. The volume of the system was made up to 1·0 ml. with saline. In the absence of inhibitor 50 per cent haemolysis of the system occurred after 30 min. of incubation at 37°. Haemoglobin values were read in a Klett-Summerson Photo-electric colorimeter using a green filter (540 mμ). Results obtained with the various inhibitors are given in Table V. The Table shows that all inhibitors of the AE system also inhibit immune haemolysis, however the concentrations of some of the inhibitors

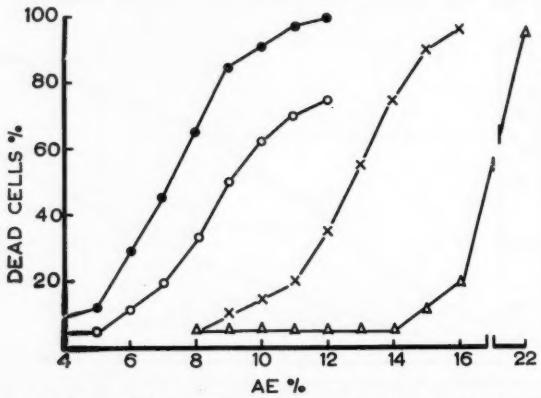

FIG. 2.—Effect of Mg^{++} on AE activity in phosphate buffer and in serum.

○———○ AE diluted in saline containing 50 per cent inactivated rabbit serum.
●———● Same but containing $1 \cdot 3 \times 10^{-3}$ M Mg^{++}.
△———△ AE diluted in phosphate saline buffer.
×———× Same but containing Mg^{++}.

needed to inhibit the latter were much smaller. Studies on the action of Ca^{++} and Mg^{++} on the inhibited systems showed that except in the case of trypan blue and Congo red, the inhibition by all other materials was reversed by $1 \cdot 3 \times 10^{-3}$ M Ca^{++} plus $1 \cdot 3 \times 10^{-3}$ M Mg^{++}. In experiments employing each cation alone it was found that while both cations were essential for complete reversal of the inhibition caused by EDTA or phosphatidyl serine, reversal of the inhibition caused by cerebroside or ethanolamine was not effected by Mg^{++} alone, and that caused by phosphatidyl inositol was obtained when either Mg^{++} or Ca^{++} were added to the system. As in the case of the AE system, addition of Mg^{++} to the immune haemolysis system markedly increased the titre of haemolysis (Mayer *et al.*, 1946).

DISCUSSION

The results show that the cytotoxic effect of anti-Ehrlich ascites tumour cells serum, as well as of immune haemolysis can be inhibited by phospholipids, some of their constituents, and by agents known to inhibit streptococcal haemolysins (Tables I–V). It is of interest that agglutination of E-cells or erythrocytes was

TABLE V.—*Effect of Ca^{++} and Mg^{++} on Inhibition of Immune Haemolysis*

Reaction mixture	Percent inhibition
Amboceptor + Complement + RBC*	0
+	
Cerebroside $2 \cdot 5 \times 10^{-5}$ M	100
+ Mg^{++}‡	100
+ Ca^{++}‡	100
+ Mg^{++} + Ca^{++}	15
Phosphatidyl inositol $6 \cdot 7 \times 10^{-5}$ M†	100
+ Mg^{++}	0
+ Ca^{++}	0
+ Mg^{++} + Ca^{++}	0
EDTA 5×10^{-4} M	100
+ Mg^{++}	70
+ Ca^{++}	0
+ Mg^{++} + Ca^{++}	0
Ethanolamine 10^{-2} M§	100
Congo red $6 \cdot 7 \times 10^{-4}$ M§	100

* Amboceptor-complement employed caused 50 per cent haemolysis after 30 min. of incubation at 37°.

† Similar results were obtained with phosphatidyl serine.

‡ $1 \cdot 3 \times 10^{-3}$ M.

§ No reversal of the inhibition was obtained by Ca^{++} plus Mg^{++} ($1 \cdot 3 \times 10^{-3}$ M of each).

unaffected by all the inhibitors tested. Since inhibition by all materials could be reversed by guinea pig serum and was not effective when the inhibitors were incubated with the tumour cells prior to the addition of AE, it was assumed that the various inhibitors acted on complement essential for the cytotoxic action of the antisera used (Flax, 1956 ; Mayer, 1958). Further studies revealed that except in the cases of trypan blue, Congo red and ethanolamine inhibition of AE, action by all other materials was reversed to a large extent by Mg^{++} but not by Ca^{++}. Under the same experimental conditions reversal of the inhibition of AE by EDTA could be obtained by either Ca^{++} or Mg^{++}. The findings that Mg^{++} alone could reverse the inhibition caused by various inhibitors are in agreement with those of Mayer *et al.*, (1946) who showed that inhibition of complement by pyrophosphate could be reversed by Mg^{++} but not by Ca^{++}. These results differ however from those of Wadsworth, Maltaner and Maltaner (1936) who showed that inhibition of complement by cephalin could be reversed by Ca^{++} alone. These apparently conflicting results cannot be explained at present however, since the methods of preparation of cephalins in the 2 cases are different, no comparison of the results is possible. The reversal of the inhibition of immune haemolysis caused by EDTA and some of the inhibitors employed also differs from that found in the AE system. While Ca^{++} alone reversed the inhibition caused by EDTA, both Ca^{++} and Mg^{++} were essential for reversal of the inhibition caused by cerebroside or phosphatidyl serine. The behaviour of various inhibitors in the presence of serum was also found to differ from that observed in saline or in phosphate buffer. The inhibition of AE action by trypan blue or Congo red which was not reversed by Mg^{++} plus Ca^{++} in salt solutions was completely reversed in the presence of inactivated serum. On the other hand inhibition caused by cerebroside or proteolipid was not affected by serum. The results obtained with the 2 immunological systems employed suggest therefore that inhibition exhibited by the various phospholipids cannot be due only to the binding of cations. This assumption is further strengthened by

the findings that Mg^{++} at concentrations causing reversal of the inhibition of AE or the immune haemolysis systems markedly potentiated the cytotoxic action of the antibodies. Therefore the reversal of the inhibition observed might have been partly due to enhancement of antibody action rather than to supplementing Mg^{++}. The fact that no stoichiometric relationship between Mg^{++} and inhibitor (cephalin, cerebroside) was found and that Ca^{++} is not effective in these systems also suggests that the mechanism of inhibition of AE by the various phospholipids is a more complex one. These findings also indicate that the systems employed are deficient in Mg^{++} (Mayer *et al.*, 1946) and that the medium in which cytotoxic tests are performed is of considerable importance for the quantitative evaluation of cytotoxicity.

As for an alternative hypothesis for the mechanism of action of the various phospholipids it is known that the membrane of mammalian cells contains phospholipids arranged in yet unknown structure (Ponder, 1948). The inhibition of AE action by phospholipids may therefore be due to competition between these inhibitors and similar substrates present in the cell membranes for some enzymatic components of complement. Cushman *et al.*, (1957) have recently described the inhibition of complement action by compounds containing basic amino acids as well as by derivatives of aromatic amino acids. These authors claim that the fact that basic as well as aromatic amino acids are involved in the inhibition points to the possibility that several components of complement are proteolytic enzymes. In this respect it has been found that polylysin inhibits both complement and plasmin action (Cushman *et al.*, 1957 ; Ginsburg *et al.*, 1952). Some of the inhibitors described by Cushman *et al.*, probably act by blocking the esterase activity of the C′1-component of complement (Becker, 1956) and thus prevent cell damage. The various phospholipids under question may also serve as substrates for one or several enzymatic components of complement (C′2 ?) the fixation of which is known to be dependent on the presence of Mg^{++} (Mayer, 1958). Inhibition of complement action may also be caused by colloidal materials which adsorb complement. Inhibition by high concentrations of cholesterol is probably due to this (Browning, 1931). The mechanism of inhibition caused by trypan blue and congo red is still not known ; it is possible however that it may be due to competition between complement and some cell membrane substrates ; such mechanism has been proposed for the inhibition of streptolysin S by trypan blue and congo red (Miyaji, 1954). The inhibition caused by ethanolamine is in agreement with the findings of Gordon, Whitehead and Wormale (1926) that complement action can be inhibited by amines. It has also been found that by blocking the free amino group of this compound with methyl groups as in the case of dimethyl ethanolamine or choline the inhibitory capacity is lost. This fact also points to the possibility that the lack of inhibition of AE by lecithin may be due to the presence of choline as an end group. On the other hand phosphatidyl ethanolamine and phosphatidyl serine may be active because of their free ethanolamine and phosphoserine group. The fact however that Mg^{++} failed to reverse the inhibition of AE by ethanolamine while that exhibited by phosphatidyl ethanolamine is easily reversible by this cation probably points to different mechanisms of action of these agents. The mechanism of inhibition of AE by cerebroside and proteolipid is not known at present. It is however possible that, although purified, these materials may be contaminated with phospholipids responsible for their inhibitory capacity. Inhibition of complement action by phospholipids may shed some light on the

role of egg yolk in the suppression of anaphylactic arthritis described by Coburn *et al.*, (1954). It is assumed that complement may take part in various immunological processes leading to cellular destruction. Such a role for complement was recently described in passive cutaneous anaphylaxis and anaphylatoxin formation (Osler, 1958). It is therefore possible that inhibition of anaphylaxis by egg yolk described by Coburn *et al.*, (1954) might have been partly due to the inhibition of complement action by cephalins present in the crude phospholipids employed by these authors. The mechanism of inhibition of anaphylaxis by lipids described by Ganley *et al.*, (1958, 1959) is probably not due to interference with complement action since these authors found that choline as well as dimethyl ethanolamine were as active as ethanolamine in suppressing anaphylaxis. In addition the small quantities of materials needed to affect anaphylaxis described by these authors also suggest that different mechanisms are involved.

The relationship between complement and phospholipid levels in various diseases is of special interest. It is known that complement levels in the nephrotic syndrome are much below normal (Ellis and Walton, 1958). The decrease in complement levels is attributed to its removal by antigen-antibody reaction taking place upon kidney cells. In this respect it is important to note that phospholipid levels in this disease are much higher than normal (see Coburn *et al.*, 1954). It is therefore tempting to assume that the low titres of complement observed may be due to the anti-complementary action of elevated phospholipid. This assumption is in agreement with the notion that lipoidal antigens as well as cephalin are anticomplementary (Eagle, 1937 ; Wadsworth *et al.*, 1936).

The findings that rheumatic fever is rarely present in patients suffering from lipaemia (Coburn *et al.*, 1954), that high consumption of egg yolk phospholipids decreases the incidence of rheumatic fever recurrences (Coburn, 1945), that alcoholic fractions obtained from egg yolk could inhibit allergic arthritis in the guinea pig (Coburn *et al.*, 1954) and that people suffering from rheumatic fever possess at the same time low levels of phospholipids and high levels of complement as well as low titres of antistreptolysin S inhibitor (a phospholipoprotein) (Chang and Coburn, 1957 ; Williams and Law, 1958 and Wallis and Viergiver, 1954), all point to the possibility that the various phospholipids may have been active by suppressing the pathological sequelae of antigen-antibody reaction mediated through complement. Finally the majority of phospholipids found active against complement also inhibit the action of streptococcal haemolysins (Ginsburg, unpublished) which points to a similar mode of action of both agents.

SUMMARY

Studies on the inhibition of the cytotoxic effect of anti-Ehrlich ascites tumour rabbit serum, and of immune haemolysis by phospholipids, some of their constituents and antihaemolytic agents is described.

The inhibition caused by the various materials (cephalins, cerebroside, proteolipid, phosphoserine, ethanolamine, Congo red and trypan blue) is effective only when the various inhibitors are preincubated with anti-serum prior to the addition of cells. Since the inhibition is reversed by guinea pig serum it is assumed that the various inhibitors act on complement essential for the cytotoxic effect of the antisera. Inhibition by the majority of materials (except Congo red,

trypan blue and ethanolamine) is reversed by Mg^{++} but not by Ca^{++}. On the other hand inhibition caused by EDTA is reversed by either Ca^{++} or Mg^{++}.

Concentrations of Mg^{++} which reverse the inhibition of complement action also markedly potentiate the cytotoxic action of antisera.

The possible role of phospholipids as complement inhibitors and their relation to cytotoxic effects of immune reactions is discussed.

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SURVEY OF PAPERS

EPSTEIN and POWELL have investigated the purifying effect of a fluorocarbon upon type 5 adenovirus grown in HeLa cell culture. The results have been assessed by electron microscopy and by tests of biological activity (p. 559).

EPSTEIN, HOLT and POWELL report further work on fluorocarbon purified type 5 adenovirus. A cytochemical study combined with electron microscopy and fluorescence microscopy has been carried out on the material. Valuable morphological information has been obtained and in addition the virus has been shown to contain a substantial quantity of deoxyribonucleic acid which was seen to be localized in the nucleoid (p. 567).

KEPPIE, COCKING, WITT and SMITH report further work on the basis of virulence of *Past. pestis*. They have immunized guinea-pigs with relatively non-toxic cell-wall material obtained as a residue after extraction with ultrasonic waves. The material has been shown to be present in organisms grown *in vitro* as well as in those grown *in vivo*. The results suggest the possibility of developing a sterile, non-toxic vaccine for immunizing man (p. 577).

MIMS studied the toxic effect on mice of non-neuro-adapted strains of influenza A introduced intracerebrally. Brain sections were treated with fluorescent antibody to influenza A at various times after the injection of such strains and the appearances compared with those produced by infection with the neuro-adapted strain, NWS. The author concludes that the toxic effect of a large dose of non-neuro-adapted virus is due to a single cycle of multiplication in many ependymal and meningeal cells, and found that this was preventable by RDE. Strain NWS, on the other hand, undergoes successive multiplication cycles in these cells and then in the parenchymal cells of the brain as well; non-neuro-adapted strains behave in this way only in host mice less than 24 hr. old (p. 586).

MIMS injected large doses of the PR8 strain of influenza A virus intravenously in mice, and then examined their livers at intervals thereafter, titrating for virus infectivity and haemagglutinating activity, and staining sections with fluorescent antibody and with haematoxylin and eosin. He found that the toxic effect ascribed to large doses of influenza virus did not occur in the absence of some increase in virus infectivity, haemagglutination and fixation of fluorescent antibody; and that preparations of virus with a low infectivity/HA ratio were non-toxic even in most massive doses (p. 593).

STUART, BIOZZI, STIFFEL, HALPERN and MOUTON, using the clearance of colloidal carbon from the blood as an indicator of phagocytic activity, examined the effect of various lipids on this function in mice. Olive oil and glyceryl trioleate were stimulatory, glyceryl mono-oleate had no effect, ethyl stearate and oleate were inhibitory (p. 599).

CHLOE TAL and LAUFER studied the induction of amyloidosis in mice injected with Freund's adjuvant or its separate constituents. Of these the *Mycobacterium tuberculosis* fraction was the most effective. The addition of mouse liver emulsion, as an isoantigen, to the adjuvant or its fractions was without effect. The spleen was the first organ to react. In some cases amyloid was found in the liver and kidneys as well (p. 605).

TYRRELL, BUCKLAND, LANCASTER and VALENTINE examined a virus isolated from *Erythrocebus patas* monkeys. They determined its size and shape by centrifugation, filtration and electron microscopy, investigated its resistance to physical and chemical agents and studied its infectivity for various host cells. They characterized the red cell receptors concerned in its haemagglutinating activity, and established its serological identity with

SV17 virus and its ability to fix complement with convalescent sera from cases of adenovirus infection in man. This last finding accorded with the agent's resemblance to human adenoviruses in every other respect (p. 610).

AGOSTINI, PERIN and FONNESU studied the influence of adrenalectomy on the fatty livers of rats induced by white phosphorus and carbon tetrachloride. Contrary to the many published reports, these authors found that adrenalectomy had little, if any, effect on the development of fatty livers (p. 617).

OLITZKI has studied deoxyribonucleic acid conjugated proteins from *Brucellae*. He has shown that these substances are highly antigenic in rabbits, giving rise to precipitating immune sera. Agar diffusion experiments show the presence of one sharp precipitation line, but none of the proteins examined were specific for a single member of the genus Brucella (p. 623).

HILL, HOWARD and GRESHAM have investigated the electrocardiographic appearances of myocardial infarction in the rat. Animals were fed on a diet known to produce infarction or were injected with isoproterenol. They show that electrocardiography can provide early evidence of infarction in the rat, the most important feature being inversion of the T wave (p. 633).

SHEFFIELD, BAUER and SYLVIA STEPHENSON investigated the action of isatin β-thiosemicarbazone and some related compounds on various pox viruses growing in embryo rabbit kidney cells in culture. They found that isatin β-thiosemicarbazone, though without effect on the infectivity of the virus suspensions, prevented the appearance in infected cells of the cytopathic effect which developed in controls. They showed that attachment of virus to cells was unaffected by the presence of the compound and concluded that its inhibitory action was effective during intracellular virus replication. The related compounds tested were ineffective (p. 638).

GINSBURG has studied the inhibition of the cytotoxic effect of rabbit anti-Ehrlich ascites tumour cell serum upon the tumour cells by phospholipids. He has shown that the inhibition is only effective when the various inhibitory materials are preincubated with anti-serum before the addition of tumour cells. Evidence is produced that the inhibitors act upon complement essential for the cytotoxic effect (p. 648).

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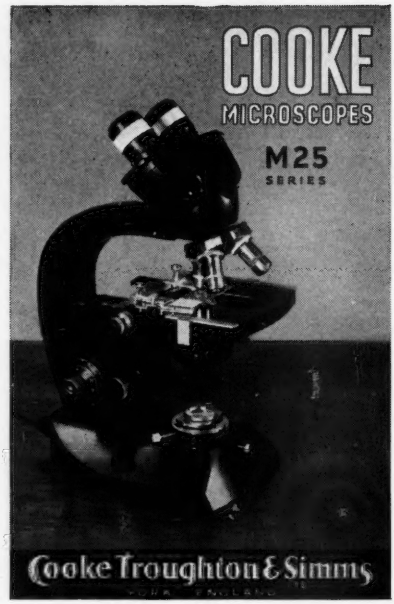

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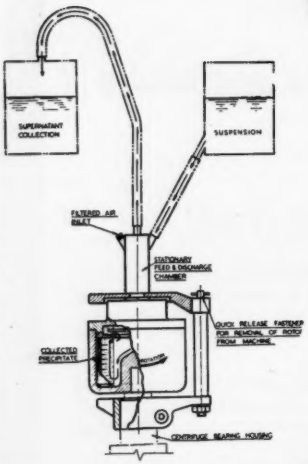

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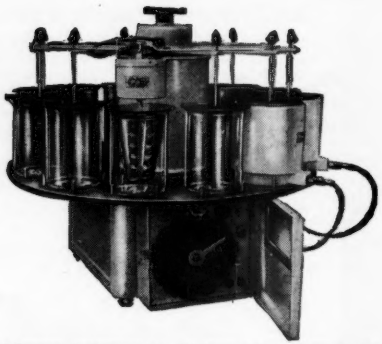

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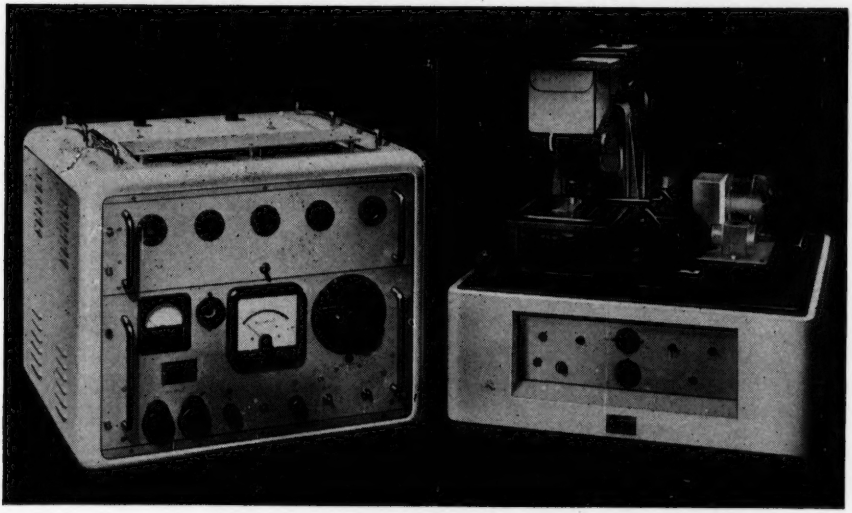

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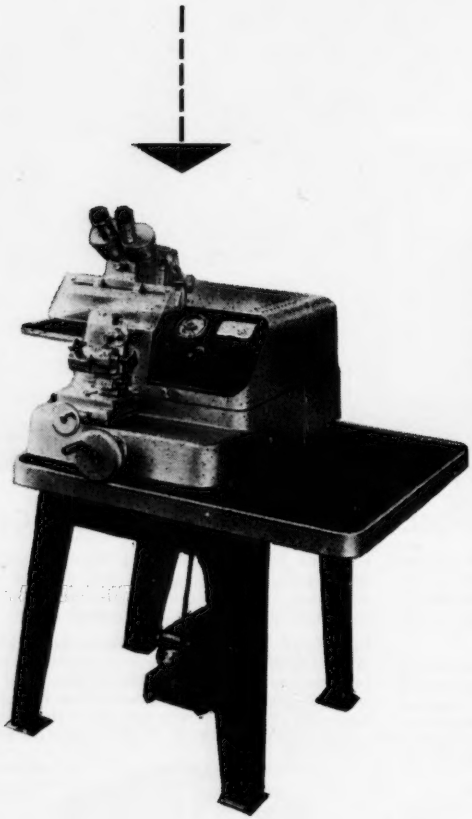

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